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ON THE FACTOR PRODUCING AGGLUTINATION OF SENSITIZED RED CELLS AND ITS RELATION TO THE AGGLUTINATION OF HEMOLYTIC STREPTOCOCCI IN RHEUMATOID ARTHRITIS SERA

BY

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HELSINKI, 1950

Helsinki 1950
Kirjapaino Oy. Liike

To my wife

PREFACE

The incentive for this work, which has been carried out in the Department of Serology and Bacteriology of the University of Helsinki, was given by the report published in the spring 1948 by H. M. Rose, C. Ragan, E. Pearce and M. I. Lipman on their investigations concerning the agglutinating capacity of rheumatoid arthritis sera against sensitized sheep cells. At the suggestion of my chief, Professor K. O. Renkonen, M. D., I started in the autumn of 1948 to study this phenomenon, which later formed the basis for also the other investigations performed in this work. I am deeply grateful to Professor Renkonen not only for his valuable advice and support but also for his unfailing and warm interest, all of which have greatly furthered my work.

I also extend my appreciative thanks to the chiefs of different hospitals who have kindly allowed me to conduct examinations of their patients. I particularly wish to mention the chief of the Kivelä Hospital in Helsinki, Professor P. Soisalo, M. D., the assistant chief of the same hospital, Mr. M. Virkkunen, M. D., and the chief of the Heinola Sanatorium for Rheumatic Diseases, Mr. V. Laine, M. D.

My sincere thanks are due also to my associates in the Department of Serology and Bacteriology of the University of Helsinki and in the State Serum Institute, of whom I particularly thank Mr. M. Tuomioja, M. D., Mr. K. Penttinen, M. D., and Mr. H. R. Nevanlinna, M. D., for their interest and valuable advice.

Misses Birgit Strömberg and Hedvig Öhberg have been of great assistance in the practical execution of my work and I thank them heartily for this. I am also indebted to all members of the staff of the Department of Serology and Bacteriology who in any way have rendered me assistance.

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Helsinki, May 1950.

Odd Wager

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CHAPTER I

REVIEW OF THE LITERATURE ON AGGLUTINATION PHENOMENA IN RHEUMATOID ARTHRITIS

A. Bacterial Agglutination

The agglutination phenomena seen in rheumatic diseases became a subject of research in the early 1930's, when Cecil, Nicholls & Stainsby (7, 33, 34) observed that the sera of persons with rheumatoid arthritis have a strong agglutinating power on certain streptococci. Cecil *et al.* isolated these streptococci (»typical strains») from the blood and synovial fluid of patients with rheumatoid arthritis and they regarded them as attenuated hemolytic streptococci. The observations made by these workers formed the initiative for active research on the subject. In 1932—1936 Dawson, Olmstead and coworkers (15, 16, 17, 18) demonstrated that rheumatoid arthritis sera agglutinate not only these »typical strains» of streptococci but also streptococci of the Lancefield group A. The positive results obtained by different investigators in agglutination tests in rheumatoid arthritis with group A streptococci vary from 36 to 94 per cent of the cases of this disease but are generally between 50 and 60 per cent [Porsman (45) 1948]. In addition to group A streptococci, hemolytic streptococci of also other Lancefield groups are agglutinated by rheumatoid arthritis sera. although not as strongly [McEwen *et al.* (28) 1935, Dawson *et al.* (14) 1936].

It was found in 1932 by Dawson, Olmstead & Boots (17) that the rheumatoid arthritis sera which agglutinate hemolytic streptococci will without exception and in nearly as high titers agglutinate also non-encapsulated pneumococci. These pneumococci were not agglutinated by the sera of rabbits immunized with

hemolytic streptococci. By absorption of the rheumatoid arthritis sera with hemolytic streptococci it was possible to remove their agglutinating action against these streptococci and to reduce but not completely remove this action against pneumococci. Absorption with pneumococci, again, effected some reduction in the agglutinating power against streptococci.

Olhagen (37) 1947 isolated from the pharynx of patients with rheumatoid arthritis some strains of enterococci (*Streptococcus faecalis*) which he found to be more strongly agglutinated by most rheumatoid arthritis sera than by other sera.

Oker-Blom (35, 36) 1948–49 demonstrated that rheumatoid arthritis sera exerted a nearly as strong agglutinating action against several different strains of *Staphylococcus aureus* as against group A streptococci. By absorption of these sera with staphylococci the agglutinating power against both streptococci and staphylococci was brought to disappear.

Numerous investigators have studied the nature of the streptococcal agglutination and its specificity in rheumatoid arthritis but the findings are contradictory in many respects. It has been observed that the streptococcal agglutinins disappear from the sera of patients with rheumatoid arthritis as the condition improves and that a certain correlation probably exists between the degree of arthritic changes and the agglutination titer [Cecil, Nicholls & Stainsby (7) 1931, Nicholls & Stainsby (33) 1933] and between the duration of the disease and the agglutination titer [Dawson, Olmstead & Boots (16) 1932, Cecil & de Gara (6) 1946, Porsman (45) 1948]. On the other hand, fluctuations have been observed in the agglutination titer from week to week without concurrent alterations in the clinical condition [Cox & Hill (10) 1934, Short, Dienes & Bauer (47) 1937, Porsman (45) 1948]. It has been stated that the streptococcal agglutination is not positive until four to six months have passed from the onset of the disease [Edström & Winblad (20) 1947] and that the prognosis for the patient is more unfavourable in cases with positive than in those with negative streptococcal agglutination [Boots, Lipman, Coss & Ragan (2) 1949]. In a number of studies no definite correlation was found to exist between the streptococcal agglutination and the sedimentation rate [Dawson *et al.* (16) 1932, Cecil *et al.* (6) 1946, Kalbak

(26) 1946] but in other investigations such a correlation was distinct [Winblad & Edström (59) 1948] or statistically significant [Thulin (52) 1948]. It has been observed that a high agglutination titer for streptococci is rarely seen in association with a high antistreptolysin titer [Boots *et al.* (2) 1949], which latter generally is not higher, or at least not significantly higher, in cases of rheumatoid arthritis than in normal material [*i.a.* Myers & Keefer (32) 1934, Bunim & McEwen (5) 1940, Kalbak (24, 25, 26) 1942—1946, Porsman (45) 1948, Boots *et al.* (2) 1949]. The factor in rheumatoid arthritis sera responsible for the agglutination of streptococci belongs to the β-γ-globulin fraction [Boots *et al.* (2) 1949].

Specificity of Streptococcal Agglutination in Rheumatoid Arthritis

The question of specificity of the streptococcal agglutination in rheumatoid arthritis is frequently discussed. Cecil and coworkers (6, 7, 33, 34) regarded the reaction as a specific, true antigen-antibody reaction, such as for instance the Widal reaction for typhoid fever, and considered that it supported their theory of the streptococcal etiology of rheumatoid arthritis. Dawson *et al.* (16) gained the impression that the streptococcal agglutination in rheumatoid arthritis is of a somewhat different nature from the ordinary immune agglutination reactions which occur during the course of specific infectious diseases and they pointed to the possibility of its being produced by increased activity of the natural agglutinins in human sera. The natural agglutinins had been earlier studied by Gibson (21) 1930, who ascertained that they are more thermolabile than immune agglutinins but less so than the complement. According to Dawson *et al.* (16) rheumatoid arthritis agglutinins occupy a similar position between the complement and the immune agglutinins. In some of their later papers, however, these workers considered it probable that streptococcal agglutination in rheumatoid arthritis is a specific immune reaction produced by infection with *Streptococcus haemolyticus*.

Kalbak (26) 1946 expressed the opinion that nothing as yet disproves the specificity of the reaction in rheumatoid arthritis and that a positive streptococcal agglutination reflects the spreading of the streptococci in the organism and the antistreptolysin titer reflects

the toxin-producing capacity of these bacteria. Rheumatoid arthritis, he suggested, is possibly a chronic streptococcal sepsis.

In a number of other publications the specificity of the reaction seems to be regarded as possible [*e.g.* Packalén (41) 1943, Winblad & Edström (59) 1948].

Particularly in recent years the opinion has increasingly won ground that the reaction in rheumatoid arthritis is nonspecific. Packalén (42) 1948 suggested that the entirely nonspecific alteration in the serum which gives rise to the increased tendency to aggregation of the red cells, as manifest in the increased sedimentation rate, may also increase the tendency of the streptococci constituting the antigen to agglutinate under the influence of specific antibodies in the agglutination test. Olhagen (37) 1947 considered it hardly probable that the streptococcal agglutination, any more than the enterococcal agglutination, would be a specific immune reaction in rheumatoid arthritis. Oker-Blom (36) 1949 was of the opinion that inasmuch as by absorption with staphylococci he was able to remove the capacity of rheumatoid arthritis sera to agglutinate both staphylococci and streptococci, and as corresponding tests with streptococcal and staphylococcal immune sera do not indicate the existence of common antigens in these bacteria, it is difficult to solve whether the question is of specific agglutinins or merely of a nonspecific agglutinating property of the rheumatoid arthritis sera. In the opinion also of Porsman (45) 1948 the problem of the specificity of streptococcal agglutination in rheumatoid arthritis has not yet been definitely solved.

Winblad & Edström (20, 59) 1947—1948 called attention to the length of time which passes from the onset of rheumatoid arthritis and rheumatic fever before the streptococcal agglutination becomes positive. In this respect the streptococcal agglutinins in rheumatoid arthritis differ from ordinary antibodies. They considered it probable that in the course of the disease the streptococci form a new antigen localized on the surface of the bacterial body. This new antigen, called the L antigen (living antigen), would in turn stimulate the host organism to produce specific L agglutinins, characteristic of which is that they are present only in rheumatoid arthritis and the late stages of rheumatic fever and that they react when living streptococci are used as antigen.

Working on the hypothesis that the antigen structure of the strepto-

coccus resembles in part that of the bacteria in the colon group, Thulin & Vahlne (53) and Thulin (51, 52) 1946–1948 demonstrated in the streptococci two more forms of antigen in addition to the above mentioned superficial L antigen associated with living streptococci, *i.e.* the thermostable somatic O antigen and the thermolabile K antigen (capsular antigen) which inhibits O agglutination. In their opinion rheumatoid arthritis sera probably contain both O agglutinins and L agglutinins specific for group A streptococci. The L agglutinins react when living streptococci are used as antigen, whereas the O agglutinins react with a streptococcal antigen autoclaved at a temperature of + 120° C. The O agglutinins also occur in the sera of patients with rheumatic fever and nephritis.

B. Agglutination of Collodion Particles

In 1946–1947 Wallis (56, 57, 58) found that several rheumatoid arthritis sera agglutinate not only hemolytic streptococci and non-encapsulated pneumococci but also collodion particles. The agglutination titers of the sera for streptococci and collodion particles were not consistently correlated. The collodion agglutinating factor was destroyed when the sera were kept at a temperature of + 65° C. for 30 minutes and it resided in the globulin fraction of the serum. Wallis believed the agglutination of the collodion particles to be analogous to certain nonspecific flocculation phenomena, such as the Takata-Ara, colloidal gold, cephalin-cholesterol, and formol-gel reactions and considered it to be related to the elevated globulin level of the rheumatoid arthritis sera. The sera of rabbits immunized with group A streptococci and of patients with scarlet fever did not agglutinate collodion particles; the same was true of most of the normal human sera tested. Absorption with streptococci removed the ability of the rheumatoid arthritis sera to agglutinate streptococci, and their capacity to agglutinate collodion particles was reduced but not abolished. Absorption with collodion particles reduced the agglutination titer of a rheumatoid arthritis serum for streptococci from 1:640 to 1:160. When rheumatoid arthritis sera were absorbed with the non-encapsulated *Pneumococcus*, *Staphylococcus albus* or *Streptococcus viridans*, their agglutinating action against collodion particles was reduced, but not as consistently as when absorption was made with *Streptococcus haemolyticus*.

The property of the rheumatoid arthritis sera to agglutinate streptococci and pneumococci was interpreted by Wallis as a capacity to intensify in a nonspecific manner the action of the weak streptococcal and pneumococcal agglutinins already normally present in the human sera. He thought it probable that this property is related to the power of rheumatoid arthritis sera to agglutinate collodion particles.

C. Hemagglutination Phenomena

Incidental to the performance of the complement-fixation test, at least three investigators (29, 46, 54) have independently observed that some human sera cause strong agglutination of the red cells of sheep sensitized with small amounts of the homologous amboceptor. The first report on this phenomenon was by Meyer (29) 1922. He found that the serum of a patient with cirrhosis of liver strongly agglutinated sensitized sheep and guinea pig cells but not beef cells. Similarly, the serum of a patient with chronic bronchitis was capable of strongly agglutinating sensitized sheep and human cells but not of exerting this action against sensitized beef cells or against typhoid or dysentery bacilli which had been sensitized with their homologous immune sera. Meyer was of the opinion that this serum intensified the specific agglutination between the red cells and the homologous amboceptor used for sensitization. He demonstrated by absorption experiments that the agglutination-intensifying substance (»Agglutinationsförndernde Substanz») in the chronic bronchitis serum was not adsorbed to normal sheep cells but that this was the case, on the other hand, with sensitized sheep cells in an increasing degree the greater the dose of amboceptor used for sensitization. By using for absorption the sensitized red cells of one species of animal he was able to remove the agglutination-intensifying property of a patient's serum also against the red cells of another species. The factor which intensified the agglutination was relatively thermostable and was destroyed only when the serum was kept at +70°C. for 30 minutes. It was also destroyed by cobra venom, which fact, according to Meyer, pointed to the lipoid nature of the factor. He suggested that this factor possibly belonged to the third component of the complement.

A similar capacity to agglutinate sensitized red cells of sheep was

observed by Waaler (54) 1940 in a rheumatoid arthritis serum. The agglutination titer of this serum for sensitized sheep cells was directly proportionate to the dose of homologous rabbit anti-sheep cell amboceptor used for sensitization of the cells. On the other hand, it was possible to effect a manifold increase (up to 32-fold) in the agglutination titer of the homologous amboceptor serum for sheep cells by adding to each tube in the amboceptor dilution series a small fixed dose of the rheumatoid arthritis serum in question. Waaler called the factor responsible for the phenomenon in rheumatoid arthritis serum the »agglutination activating factor», and it was his opinion that this factor intensifies the specific hemagglutination in the same manner as Meyer's factor. The effect of this agglutination activating factor resembled the conglutination phenomenon described in 1906—1909 by Bordet, Gay and Streng (3, 4, 48). These investigators had found among others in beef serum a factor (conglutinin) which in the presence of the complement had brought about the aggregation of red cells sensitized with the homologous amboceptor. Waaler's agglutination activating factor did not require the presence of the complement and he therefore concluded that its mode of action could not be identical with that of conglutinin. However, the properties of the agglutination activating factor pointed to relationship with the intensifying action on specific agglutination found in the sera of the guinea pig and certain other animals by Dean (19) 1911 and Olsen (39) 1922. — Waaler found a definite agglutination activating action in one-third of 77 rheumatoid arthritis sera; the remaining two-thirds as well as the 202 control sera had a weak intensifying action. He regarded that the phenomenon was not sufficiently characteristic of rheumatoid arthritis to permit its use in the diagnosis of this disease. However, he believed that the sera of mainly those patients with rheumatoid arthritis whose condition was severe contained the agglutination activating factor. No correlation was seen by him between the agglutination activating action on the one hand, and the sedimentation rate, the agglutinating power for hemolytic streptococci and the blood groups on the other hand. The agglutination activating factor belonged to the globulin fraction of the serum and there was no correlation to the amount of globulin, and Waaler therefore suggested that the question was one of qualitative changes in the globulin fraction. The factor was quite thermostable, as it could

stand a temperature of +60°C. for at least 30 minutes. As the sera in the control series also had a weak tendency to intensify the agglutination he was of the opinion that we probably have in rheumatoid arthritis a case of intensification of a property already present in normal serum. Absorption with sensitized sheep red cells reduced the agglutination activating power in rheumatoid arthritis sera to some extent but the active principle could not be removed completely. No reduction whatsoever was seen in this property when nonsensitized sheep red cells were employed for the absorption.

In 1948 Rose, Ragan, Pearce & Lipman (46) again drew attention to this phenomenon, apparently unaware of the findings of Meyer and Waaler. The principal points in the observations made by Rose *et al.* were the following. Sheep cells sensitized with a small amount of the homologous rabbit anti-sheep cell amboceptor are agglutinated by most human sera more strongly than are nonsensitized sheep cells. In the sera of patients with rheumatoid arthritis in an active stage this difference in the titer between sensitized and nonsensitized sheep cells (*i.e.* the differential agglutination titer, designated in the present work as DAT) was in the majority of cases $\geqq 16$, and in sera taken at an inactive stage of the disease and in control sera it was < 16. As an example, if the agglutination titer of a serum under test was 1 : 2048 for sensitized sheep cells and 1 : 32 for nonsensitized sheep cells, the DAT value was: $\frac{2048}{32} = 64$. No definite correlation was found between the DAT and the agglutination of group A streptococci by rheumatoid arthritis sera. It is true that in cases with a high DAT the streptococcal agglutination was generally positive, but in numerous cases with a high DAT it was negative or doubtful. These authors therefore considered it probable that the serum factor which causes the DAT is different from the factor which produces the agglutination of group A streptococci by rheumatoid arthritis sera. No direct correlation was found to exist between the DAT and the agglutination of collodion particles, nor between the DAT and the sedimentation rate. Electrophoretic fractionation indicated that the serum factor responsible for the DAT belongs to the β-γ-globulin fraction. According to the observations of Rose *et al.* the DAT reflects the activity of the disease process in rheumatoid arthritis and they suggested its probable practical significance as a serodiagnostic test. They designated this test as the

»differential sheep cell agglutination test», which in the present work is briefly called the »Waaler-Rose test»[1], irrespective of the designations used by the different investigators.

Sulkin, Pike & Goggeshall (49) 1949 also reached the conclusion that the DAT reflects the clinical severity of rheumatoid arthritis and believed that the Waaler-Rose test would be of limited value as an aid in the diagnosis of active rheumatoid arthritis of mild severity. Jawitz & Hook (23) 1949 obtained a »positive» Waaler-Rose test value (DAT $\geqq$ 16) not only in several cases of rheumatoid arthritis in an active stage but also in a case of Marie-Strümpell arthritis. In two cases of cirrhosis of liver the DAT values were higher (DAT 8 and 16) than in other cases in the series examined. They obtained the impression that elevated DAT values occur chiefly in severe cases of rheumatoid arthritis. They observed these values in six patients over a period of six months and found that the course of the disease was reflected in the values in the form of a reduction as the condition improved — far in advance, in fact, of any recognizable change in the sedimentation rate. Consequently the Waaler-Rose test was believed by these authors to be of possible value in estimating therapeutic results in rheumatoid arthritis. The factor in the serum responsible for the DAT could not be absorbed with guinea pig kidney, which has been found to adsorb the heterophilic agglutinins for sheep red cells in the sera of healthy persons and those with serum sickness (11). Neither could it be absorbed with boiled beef cells, which can adsorb the heterophilic agglutinins for sheep red cells in infectious mononucleosis sera (12). The factor retained its action at a temperature of −70°C. for at least three months but was rapidly destroyed if the serum became infected.

Miller, Lynch & Landsbury (30) 1949 obtained a »positive» Waaler-Rose test in 67 per cent of all the cases of active rheumatoid arthritis studied, but found a similar result also in one case of osteoarthritis and four cases of latent syphilis. No correlation was seen by them between the DAT values and the clinical activity of rheumatoid arthritis, nor between the DAT values and the sedi-

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[1] The designation »Rose test» was used in a preliminary report on the present work (55). However, in view of the merits of Waaler as the first observer of the phenomenon with rheumatoid arthritis sera, the term »Waaler-Rose test» is deemed more justified.

mentation rate. The authors regarded that the Waaler-Rose phenomenon was not specific for rheumatoid arthritis to a degree sufficient to provide diagnostic support.

By modification of the original technique of Rose *et al.* in such a manner that the normal sheep cell agglutinins in the sera were first absorbed with nonsensitized sheep cells, Heller, Jacobson & Kolodny (22) 1949 were able to effect certain definite improvements in the test. In the first place the agglutination titers of such absorbed sera for sensitized sheep cells indicate directly the concentration of the factor associated with rheumatoid arthritis and it therefore is not necessary to indicate the test result as a differential titer. Secondly this modified test was »positive» (*i.e.* agglutination of sensitized cells occurred in serum dilution $\geqq 1 : 28$) in 90 per cent of cases of rheumatoid arthritis, whereas the original Waaler-Rose test carried out at the same time was »positive» in 60 per cent only. A »positive» modified test was obtained not only with rheumatoid arthritis sera but also with two hepatitis sera. Correlation was not always found between the modified test and the clinical severity of the disease, but on the other hand the test was not »positive» in a single inactive case of rheumatoid arthritis. A serious limitation of the test as a diagnostic tool was that the test apparently became »positive» only when X-rays began to show the joint changes characteristic of rheumatoid arthritis. It was not possible to remove the agglutinating factor for sensitized sheep cells by absorption with nonsensitized sheep cells or with boiled ox cells. This and the fact that the factor belongs to the β-γ-globulin fraction were understood by Heller *et al.* to point to the antibody nature of the factor, and they suggested the necessity of studying whether the factor is a result of immunization by an etiological agent or if it is analogous to the nonspecific antibody of the complement-fixation reaction which is almost always associated with syphilis.

Swartz & Schlossmann (50) 1949 obtained a »positive» Waaler-Rose test in 33 per cent of rheumatoid arthritis cases. In an attempt to solve whether infection might play a role in the production of the factor in rheumatoid arthritis serum responsible for the DAT they injected different bacteria in test animals. The agglutination titer of rabbit sera for both nonsensitized and sensitized sheep cells increased considerably after such immunization with enterococci. A similar, although weaker, effect was obtained with

a strain of pneumococci (type 52) but this was not the case with group A streptococci or any of the other bacteria used. Absorption with the bacterial strains which had been used to produce the hemagglutinins did not eliminate these hemagglutinins from the animal sera. In most cases it was possible to remove the hemagglutinating power for both nonsensitized and sensitized sheep cells by absorption with nonsensitized sheep cells. In this respect these sera differed from rheumatoid arthritis sera, from which only the capacity to agglutinate nonsensitized sheep cells was removed by absorption with nonsensitized sheep cells, whereas the agglutinating power for sensitized sheep cells remained unchanged. The latter property was removed from the rheumatoid arthritis sera by absorption with highly sensitized sheep cells. It was the opinion of these workers that further development of the Waaler-Rose test technique would render the reaction serodiagnostically serviceable.

Electrophoretic serum analyses carried out by Olhagen (38) 1949 indicated an increased amount of γ-globulin in rheumatoid arthritis sera but showed no quantitative correlation between the level of γ-globulin and the DAT value. A »positive» Waaler-Rose test was obtained by them not only in rheumatoid arthritis but also in certain cases of acute disseminated lupus erythematosus, in myeloma and in severe liver lesion, in all of which the γ-globulin level of the serum was elevated. Olhagen referred to the thermostable anticomplementary effect of the sera in myeloma, chronic liver lesion and rheumatoid arthritis and stressed the necessity of studying the interrelation of these diseased conditions.

Ouchterlony & Jonsson (40) 1949 reported a »positive» Waaler-Rose test in 60 per cent of cases of rheumatoid arthritis.

A report recently published by Pike, Sulkin & Goggeshall (44) 1949 describes the results of supplemental investigations on the nature of the serum factor which agglutinates sensitized sheep cells. This factor (»the rheumatoid factor») was not connected with the agglutinating power of rheumatoid arthritis sera for nonsensitized sheep cells, which was not stronger than in other sera. Neither was it possible to explain the effect of the »rheumatoid factor» to be a result of a simple additive effect of the normal sheep cell agglutinins in the rheumatoid arthritis serum and in the sensitizing amboceptor serum. The »rheumatoid factor» could not be absorbed with nonsensitized sheep cells but probably was adsorbed to some extent to

sensitized sheep cells. However, absorption with sensitized sheep cells did not in a single case reduce the agglutination titer of the rheumatoid arthritis sera for sensitized sheep cells more than to one-fourth of the original titer. It was not possible to remove the »rheumatoid factor» from the sera by extraction with lipoid solvents. The factor had no effect on the hemolytic system. In addition to sheep cells sensitized with rabbit anti-sheep cell amboceptor, the following cells sensitized with homologous rabbit amboceptors were affected by the »rheumatoid factor»: goat cells sensitized with anti-goat cell and anti-sheep cell amboceptor, beef cells sensitized with anti-beef cell amboceptor, and sheep cells sensitized with anti-guinea pig kidney amboceptor. The »rheumatoid factor» had no effect on the following sensitized cells: sheep cells sensitized with the serum of a patient with infectious mononucleosis or with normal human serum, 0 cells sensitized with rabbit anti-0 cell amboceptor, rat cells sensitized with normal dog serum or with rabbit anti-rat cell amboceptor. The authors therefore came to the conclusion that to indicate the action of the »rheumatoid factor» it was necessary to have, on the one hand, sheep cells or cells serologically related to them, and, on the other hand, antibodies for certain antigenic components of the sheep cells, inasmuch as all sera containing sheep cell agglutinins did not have a corresponding sensitizing power in these tests. — Absorption of the rabbit anti-sheep cell amboceptor with guinea pig kidney and boiled beef cells did not reduce the agglutination titer of the amboceptor for nonsensitized sheep cells nor its sensitizing power in the Waaler-Rose test, whereas absorption with goat cells completely removed the agglutinating power against goat cells and reduced the agglutination titer of the amboceptor for nonsensitized sheep cells from 1 : 1280 to 1 : 320 and decreased its sensitizing power in the Waaler-Rose test. No direct parallelism could be seen between the DAT and the agglutination of group A streptococci by rheumatoid arthritis sera. It was possible by absorption with streptococci to remove from the rheumatoid arthritis sera the agglutinating power against streptococci but not the »rheumatoid factor». This led the authors to the conclusion that there is no connection between these two properties. A common feature in those antigen-antibody systems in which the effect of the »rheumatoid factor» was seen was that the agglutinins were produced by immunization of rabbits. An exception to this rule was the rabbit anti-0 cell amboceptor, as the 0

cells sensitized with this amboceptor were not more strongly agglutinated by rheumatoid arthritis sera than nonsensitized 0 cells.

Penttinen (43) 1950 made the observation that a rheumatoid arthritis serum (DAT 2048) exerted no action on virus hemagglutination (influenza A virus, strain P.R.8, chicken cells).

CHAPTER II

THE PROBLEMS AND THE AIM OF THE PRESENT WORK

Originally the object of the following work was to study the adaptability of the Waaler-Rose test for a routine method in serological diagnosis of rheumatoid arthritis. Examination of the literature on the peculiar agglutination phenomena seen in rheumatoid arthritis gave an incentive for more far-reaching investigation. Many of the observations reported in the literature seemed to point to a close correlation between the different agglutination phenomena observed in rheumatoid arthritis. The attention was drawn in particular to the investigations by Wallis (56, 57, 58) and Waaler (54). Wallis ascribed the capacity of rheumatoid arthritis sera to agglutinate hemolytic streptococci and non-encapsulated pneumococci to a peculiar property of these sera to intensify in a nonspecific manner the action of the weak specific agglutinins which probably are present in most human sera. Waaler ascribed the capacity of the rheumatoid arthritis sera to agglutinate sheep red cells sensitized with a homologous rabbit anti-sheep cell amboceptor to the presence in these sera of an »agglutination activating factor» which intensifies the specific agglutination between sheep cells and the homologous sensitizing amboceptor. The analogy between these two explanations is obvious. Accordingly, Wallis and Waaler had independently of each other and by different arguments reached in principle the same opinion that rheumatoid arthritis sera contain a factor which intensifies the specific agglutination.

These reports in the literature gave rise to the question whether or not all the agglutination phenomena seen in rheumatoid arthritis are produced by one and the same agglutinating factor in the serum.

It seemed possible that in such a case a closer study of the nature of the Waaler-Rose phenomenon might provide an explanation for the other agglutination phenomena seen in rheumatoid arthritis. In view of the great importance ascribed by many authors to streptococcal agglutination as evidence pointing to the streptococcal etiology of rheumatoid arthritis it was considered important, in setting out upon the present work, to give special attention to the study of the possible relation between the Waaler-Rose phenomenon and the streptococcal agglutination phenomenon in rheumatoid arthritis.

The chief questions for which a solution was sought in the work described in the following pages were:

1. Is the occurrence of the Waaler-Rose phenomenon specific for rheumatoid arthritis sera or does this reaction also occur with some other human sera?

2. What conclusions may be drawn from the hemagglutination and absorption experiments regarding the nature and mode of action of the serum factor responsible for the Waaler-Rose phenomenon?

3. Does this serum factor play a role in the agglutination of hemolytic streptococci by rheumatoid arthritis sera?

PERSONAL INVESTIGATIONS

CHAPTER III

THE WAALER-ROSE TEST WITH RHEUMATOID ARTHRITIS SERA AND OTHER HUMAN SERA

By means of the tests described in this chapter an attempt was made to discover how specific the occurrence of the Waaler-Rose phenomenon is for rheumatoid arthritis sera. Attention was also paid to possible correlation of this phenomenon to the duration of the disease and the sedimentation rate.

A. Technique of the Waaler-Rose Test

The main features of the technique used by Rose *et al.* (46) were followed in the Waaler-Rose tests performed in this work. From the serum to be studied, which had been inactivated at +56°C. for 30 minutes, two parallel series of dilutions were made with 0.9 per cent saline solution in the ratios 1 : 4, 1 : 8, 1 : 16 and so on, placing 0.5 ml. of the dilution in each tube. Into each tube of one of the dilution series was added 0.5 ml. of sensitized sheep red cell suspension, and similarly into each tube of the other series 0.5 ml. of nonsensitized sheep red cell suspension. The final dilution series for each serum was therefore 1 : 8, 1 : 16, and so on. The control was a tube containing 0.5 ml. of sensitized sheep red cell suspension and 0.5 ml. of saline solution. The tubes were kept in the water-bath at +37°C. for one hour and then overnight in the refrigerator. The agglutination readings were made on the following day by inverting each tube twice and looking against a lamp with the naked eye. The highest dilution of serum which still caused one-plus agglutination was regarded as the agglutination titer (see table 1).

The Waaler-Rose test value was determined by dividing the reciprocal value of the agglutination titer for sensitized sheep cells by the reciprocal value of the agglutination titer for nonsensitized sheep cells. If, for instance, the serum under test agglutinated sensitized sheep cells in titer 1 : 2048 and nonsensitized sheep cells in titer 1 : 16, the Waaler-Rose test value was $\frac{2048}{16} = 128 =$ differential agglutination titer (DAT). Table 1 shows the Waaler-Rose tests carried out with one rheumatoid arthritis serum and one normal serum.

The Sheep Red Cells and the Homologous Amboceptor. — The sheep red cells required for the Waaler-Rose test were obtained by centrifugating defibrinated sheep blood and then washing the cells three times in a large amount of saline. The washed cells were always used on the same day. The defibrinated sheep blood was immediately discarded if the slightest color indicating hemolysis of the cells was seen in the saline. The usual rabbit anti-sheep cell amboceptor sera employed for routine Wassermann reactions were used for sensitization of the red cells.

The Sensitized Sheep Cell Suspension. — To a 2 per cent suspension of washed cells in saline solution was added an equal volume of amboceptor dilution, and this 1 per cent suspension of sensitized cells was immediately added to the dilution series made of the patient's serum. Prior to the sensitization of the cells the *titration of the amboceptor* was carried out as follows:

A serial dilution 1 : 200, 1 : 400, 1 : 600, 1 : 800 and so on was made from the amboceptor serum, placing in each tube 0.5 ml. of the dilution. To each tube was added 0.5 ml. of the 2 per cent suspension of sheep cells and 1.0 ml. of saline solution. The tubes were kept in the water-bath at +37°C. for one hour and in the refrigerator overnight, after which the agglutination reading was taken as described above for the Waaler-Rose test. The sensitizing dose selected was 1/2 × minimum agglutinating dose (1/2 × MAD). Thus, if the highest dilution of amboceptor which was still capable of agglutination was 1 : 1000 (amboceptor serum dose 0.0005 ml.) the dilution selected as sensitizer was 1 : 2000 (amboceptor serum dose 0.00025 ml.). — Preliminary tests had shown that rheumatoid arthritis sera agglutinate sensitized sheep cells the more strongly the greater the amboceptor dose used for sensitization of the cells. It was, on the one hand, the effort to devise as sensitive a system as possible but, on the other hand, the dose of amboceptor used for sensitization should not be so great that there would be a danger of its causing agglutination alone without the presence of the patient's serum. The optimum dose corresponding to these requirements was found to be 1/2 × MAD, which thereupon was used in all the Waaler-Rose tests.

The Nonsensitized Sheep Cell Suspension. — A part of the same 2 per cent suspension of sheep cells from which the sensitized cell suspension was made was used for the nonsensitized sheep cell suspension. However, the amboceptor dilution was substituted by an equal volume of saline solution.

The Waaler-Rose test was generally performed one to three days after the taking

TABLE 1

WAALER-ROSE TEST WITH THE SERA OF A PATIENT WITH RHEUMATOID ARTHRITIS AND A HEALTHY PERSON

		Dilution of serum										Saline control	DAT
		8	16	32	64	128	256	512	1024	2048	4096		
Rheumatoid arthritis serum	NC	+++	++	−	−	−	−	−	−	−	−	−	128
	SC	++++	+++	+++	+++	+++	+++	+++	++	+	−		
Serum of healthy person	NC	+++	+	−	−	−	−	−	−	−	−	−	4
	SC	++++	+++	++	+	−	−	−	−	−	−		

NC = Nonsensitized sheep cells
SC = Sheep cells sensitized with 1/2 × MAD (minimum agglutinating dose) of amboceptor
DAT = Differential agglutination titer

Degree of agglutination:

++++ = Red cells form one solid cake, which remains mainly intact after inversion of tube.
+++ = After inversion of tube, red cell cake breaks up into several parts; liquid clear.
++ = After inversion of tube, red cells form numerous agglutinated clumps of various size; liquid cloudy.
+ = After inversion of tube, red cells form small, equal-sized agglutinated bodies, clearly discernible macroscopically.

of the blood samples. A number of sera were stored in unsterile tubes in the deep-freeze at −20°C. for over six months with hardly any change in the factor responsible for the Waaler-Rose phenomenon.

To obtain some idea of the technical errors possible in the Waaler-Rose test, the following simultaneous tests were made with one rheumatoid arthritis serum:

1. Seven parallel Waaler-Rose tests under fully identical conditions. The DAT obtained was 256 in all the tests.

2. Six parallel Waaler-Rose tests using the cells of the same sheep but three different amboceptors (two tests with each amboceptor). In five tests the DAT was 256 and in one test 512.

3. Eight parallel Waaler-Rose tests using the same amboceptor but cells from four different sheep (two tests with the cells of each sheep). The DAT values obtained were as follows: 512, 1024;

256, 512; 256, 256; 512, 512. Thus the greatest difference was 4-fold.

Therefore, taking into consideration that the parallel Waaler-Rose tests made each day with the clinical material were carried out with the same amboceptor and the cells of the same sheep, the technical error (see point 1 above) apparently has no marked effect on the comparison of such parallel tests. The error appears to affect somewhat more the comparison of Waaler-Rose tests performed with different amboceptors and the cells of different sheep (see points 2 and 3). Parallel examination of 15—20 sera was usually made each day, and one known rheumatoid arthritis serum with a high DAT and one known normal serum with a low DAT were always included as controls. The results for the entire series were rejected as undependable if the rheumatoid arthritis control serum did not give a DAT of $\geqq 32$ and the normal control serum $\leqq 8$. The DAT values obtained with one control serum might vary in determinations made at different times but the fluctuation generally was not more than 4-fold. If the amounts of serum available for study were sufficient, a control determination was made with those sera which had given a DAT of $\geqq 16$. If it again was $\geqq 16$, the value first obtained was selected as final. In a few rare cases the control determination gave a DAT of < 16. One more control was then made to determine which of the previous DAT values should be selected as final.

Regardless of the possibilities for error in the method used, which were diminished by including control sera in all the Waaler-Rose test series, the method can be regarded as sufficiently accurate if far-reaching conclusions are not made from the small differences in the DAT values in individual cases but attention is given to investigating, with a fairly large material, whether the Waaler-Rose phenomenon is characteristic of rheumatoid arthritis sera or whether it occurs also with other human sera.

B. Occurrence of the Waaler-Rose Phenomenon in a Case Material

Material

The material studied comprised 1291 cases. The rheumatic cases emanated chiefly from the ward for rheumatic diseases in the Kivelä Hospital in Helsinki and from the Sanatorium for Rheumatic Diseases in Heinola. The pathological controls employed were mostly sera sent for routine examination by various hospitals in Hel-

sinki, and the normal controls were blood donor sera submitted for blood grouping by the blood bank of the Finnish Red Cross. In the majority of clinical cases it was possible to obtain the diagnosis from the hospital records. For 346 cases in group X the diagnoses were unobtainable for various reasons.

The cases studied are classified into the following groups:

I. Rheumatoid arthritis (242 cases)

1. *Cases with hospital diagnosis of rheumatoid arthritis, polyarthritis chronica or polyarthritis chronica primaria* (221 cases).

2. *Controlled cases of rheumatoid arthritis* (132 cases from group 1 and 21 cases undergoing treatment under diagnoses other than rheumatoid arthritis). — In all these cases the hospital diagnosis was personally controlled by the author by examination of the patient and study of the hospital record, and they are classified into groups a)—d) according to findings.

a) *Typical cases of rheumatoid arthritis* (92 cases). — Particular attention was paid to the symmetry of joint symptoms and their occurrence in the middle joints of the fingers (»fusiform fingers») and to the typical ulnar deviation of the hands. The activity of the disease process was not taken into consideration in the grouping. In all the cases the anamnesis extended over one year.

b) *Cases of rheumatoid arthritis in initial stages* (16 cases). — Obvious cases of rheumatoid arthritis, with an anamnesis of less than one year. Swelling of the synovial capsules was the most prominent symptom in most cases and no distinct destructive changes in the bones were yet present.

c) *Atypical cases of rheumatoid arthritis* (18 cases).

d) *Cases on the borderline between rheumatoid arthritis and rheumatic fever* (27 cases).

The 21 cases under hospital diagnoses other than rheumatoid arthritis were found to belong to groups a)—d) as follows (DAT values stated in parentheses):

a) *Typical cases of rheumatoid arthritis.* — Hospital diagnoses: Case No. 1. — Periarthritis humeroscapularis. Arthrosis gen. amb. (DAT 16). Case No. 2. — Haematuria. Tumor vesicae. (DAT 1024). Case No. 3. — Thrombophlebitis v. fem. sin. (DAT 32). Case No. 4. — Fract. colli femoris sin. Chondromalacia patellae sin. (DAT 128). Case No. 5. — Infectio rheumatica. Granulocytopenia. (DAT 32).

c) *Atypical cases of rheumatoid arthritis.* — Hospital diagnoses: Case No. 6. — Arteriosclerosis. Hypertonia. Pruritus senilis. (DAT 128). Case No. 7. — Anaemia perniciosa. (DAT 128). Case No. 8. — Gonitis l. sin. (DAT 32). Case No. 9. — Cardiosclerosis. Angina pectoris. (DAT 128). Case No. 10. — Hepatitis acuta. Lues recens. (DAT 256). Case No. 11. — Glaucoma sec. o.a. (DAT 32).

d) *Cases on the borderline between rheumatoid arthritis and rheumatic fever.* — Hospital diagnoses: Case No. 12. — Febris rheumatica? (DAT 64). Case No. 13. — Febris rheumatica. (DAT 4). Case No. 14. — Febris rheumatica. (DAT 128). Case No. 15. — Febris rheumatica. (DAT 128). Case No. 16. — Polyarthritis subacuta. (DAT 32). Case No. 17. — Polyarthritis subacuta. (DAT 2). Case No. 18. — Rheumatismus chr. (DAT 128). Case No. 19. — Periostitis mandibulae. Vitium valv. mitralis. (DAT 256). Case No. 20. — Erythema nodosum. (DAT 4). Case No. 21. — Polyarthritis post infectionem. (DAT 16).

II. Rheumatic fever (65 cases).

In most cases the diagnosis in the hospital records was controlled by examination of the patient. The diagnoses stated in the records were febris rheumatica, infectio rheumatica or polyarthritis acuta; in two cases it was polyarthritis chronica.

III. Other »rheumatic diseases» (9 cases).

This group contained 7 cases of ischiadic syndrome, 1 case of palindromic rheumatism and 1 of rheumatic iritis.

IV. Various diseases with joint involvement (57 cases).

There were 18 cases of osteoarthritis, 3 of Still's disease, 3 of Marie-Strümpell arthritis, 1 of gonorrheal arthritis, 3 of tuberculous arthritis, 4 of Reiter's disease, 1 of acute disseminated lupus erythematosus, 1 of allergic arthritis, 1 of tabetic arthropathy, 1 of dermatomyositis, and 21 cases of unspecified diseases of the joints.

V. Various streptococcal diseases (23 cases).

Three cases of erysipelas, 2 of abscessus peritonsillaris, 1 of angina tonsillaris, 15 of tonsillitis, and 2 of subacute bacterial endocarditis.

VI. Infectious mononucleosis (14 cases).

The Paul-Bunnell test was positive in all these cases in serum dilution $\geqq 1 : 128$.

VII. Various diseases of the liver and the bile duct (30 cases).

Twelve cases of hepatitis epidemica, 6 of cholecystopathy, 4 of cholelithiasis, 7 of cholecystitis, 1 of icterus gravis e causa ignota.

VIII. Malignant tumors (18 cases).

Cases of various types of carcinoma according to hospital diagnoses.

IX. Syphilis (33 cases).

All were WR positive cases. The clinical diagnosis was not taken into consideration.

X. Various other diseases (702 cases).

Cases under treatment chiefly in the medical, surgical, otological, ophthalmological, dermatological, venereal, pediatric, gynecological and mental wards of the hospitals.

XI. Healthy persons (98 cases).

Results

The results of the Waaler-Rose tests carried out with the above case material are shown in table 2.

It will be seen from table 2 that when a DAT value of 16 was used as the borderline, the Waaler-Rose test was »positive» (DAT $\geqq$ 16) in 59 per cent of the cases in which the hospital diagnosis was rheumatoid arthritis or polyarthritis chronica (primaria), in 70 per cent of the controlled typical cases of rheumatoid arthritis, in 56 per cent of the initial and in 39 per cent of the atypical cases of rheumatoid arthritis, and in 44 per cent of the cases regarded as being on the borderline between rheumatoid arthritis and rheumatic fever. However, it was »positive» also in 6.1 per cent of the cases of rheumatic fever, 22 per cent of other »rheumatic diseases», 3.5 per cent of various diseases with joint involvement, 10 per cent of various liver and bile duct diseases, 28 per cent of malignant tumors, 3.0 per cent of syphilis, 5.1 per cent of various other diseases, and 1.0 per cent of the healthy persons. It therefore seems probable that when the DAT value of 16 is used as the borderline, the occurrence, at least with the technique employed in the present work, of a »positive» Waaler-Rose test is not sufficiently specific for rheumatoid arthritis sera to give the test any great diagnostic significance.

On the other hand it will be noted that if only those cases in which the DAT value is $\geqq$ 32 are regarded as »positive», the incidence of »positive» tests in cases with a hospital diagnosis of rheumatoid

TABLE 2

DISTRIBUTION OF THE DIFFERENTIAL AGGLUTINATION TITERS OF THE CASE MATERIAL

	D A T													Total No.of cases	DAT≧16		DAT≧32	
	1	2	4	8	16	32	64	128	256	512	1024	2048	4096		Cases	%	Cases	%
I. Rheumatoid arthritis														242				
1. Hospital diagnosis of rheumatoid arthritis	5	22	38	25	30	29	29	21	11	3	7	1	0	221	131	59	101	46
2. a) Controlled typical cases	0	8	10	10	8	21	12	9	6	2	5	1	0	92	64	70	56	61
b) » cases in initial stages	0	3	2	2	4	2	1	0	0	1	1	0	0	16	9	56	5	31
c) » atypical cases	1	2	6	2	2	2	1	2	0	0	0	0	0	18	7	39	5	28
d) » borderline cases rh.arthritis-rh.fever	0	5	6	4	2	3	3	4	0	0	0	0	0	27	12	44	10	37
II. Rheumatic fever	3	24	21	13	2	0	0	1	1	0	0	0	0	65	4	6.1	2	3.1
III. Other »rheumatic diseases»	0	4	2	1	1	0	0	1	0	0	0	0	0	9	2	22	1	11
IV. Various diseases with joint involvement	2	22	18	13	1	0	1	0	0	0	0	0	0	57	2	3.5	1	1.7
V. Various streptococcal diseases	2	10	9	2	0	0	0	0	0	0	0	0	0	23	0	0	0	0
VI. Infectious mononucleosis	5	3	3	3	0	0	0	0	0	0	0	0	0	14	0	0	0	0
VII. Various liver and bile duct diseases	0	9	13	5	2	1	0	0	0	0	0	0	0	30	3	10	1	3.3
VIII. Malignant tumors	1	3	4	5	3	0	1	0	1	0	0	0	0	18	5	28	2	11
IX. Syphilis	3	6	11	12	1	0	0	0	0	0	0	0	0	33	1	3.0	0	0
X. Various other diseases	60	230	269	107	23	5	4	2	2	0	0	0	0	702	36	5.1	13	1.8
XI. Healthy persons	13	39	36	9	0	0	1	0	0	0	0	0	0	98	1	1.0	1	1.0

DAT = Differential agglutination titer

arthritis or polyarthritis chronica (primaria) will decline by 23 per cent (from 131 cases to 101), in the controlled, typical cases of rheumatoid arthritis by 12 per cent only (from 64 to 56 cases), in initial cases of rheumatoid arthritis by 45 per cent (from 9 to 5), in atypical cases of rheumatoid arthritis by 29 per cent (from 7 to 5), and in the borderline cases between rheumatoid arthritis and rheumatic fever by 17 per cent (from 12 to 10 cases). The decline in the other disease groups varied from 50 to 100 per cent (average decline 67 per cent, *i.e.* from 54 to 21 cases). This means that when the borderline is set at DAT 32 the Waaler-Rose test was »positive» in 46 per cent of the cases with a hospital diagnosis of rheumatoid arthritis or polyarthritis chronica (primaria), in 61 per cent of the controlled typical cases of rheumatoid arthritis, 31 per cent of the initial cases, 28 per cent of the atypical cases, 37 per cent of the borderline cases between rheumatoid arthritis and rheumatic fever, 3.1 per cent of the cases of rheumatic fever, 3.3 per cent of various liver and bile duct diseases, 11 per cent of other »rheumatic diseases» and of malignant tumors, and less than 3.0 per cent in all the other groups.

In consideration of this finding it would seem that a DAT value of 32 is more practicable than DAT 16 for the borderline between a »positive» and a »negative» Waaler-Rose test. Therefore the term »positive» Waaler-Rose test as used henceforth in this work designates a DAT value of $\geqq$ 32.

A »positive» Waaler-Rose test was obtained in the following cases in groups other than rheumatoid arthritis:

Rheumatic fever: Case No. 1 (♀ 18 yrs.). — Febris rheumatica. Convalescentia p. hepatitidem. (DAT 128). Case No. 2 (♀ 26 yrs.). — Myocarditis p. febrem rheumaticam. Insuff. valv. mitralis. (DAT 256).

Other »rheumatic diseases»: Case No. 3 (♂ 45 yrs.). — Syndroma ischiadica l.dx. (DAT 128).

Various diseases with joint involvement: Case No. 4 (♂ 51 yrs.). — Arthrosis deformans coxae sin. (DAT 64).

Various liver and bile duct diseases: Case No. 5 (♀ 36 yrs.). — Cholecystitis ac. (DAT 32).

Malignant tumors: Case No. 6 (♀ 62 yrs.). — Carcinoma mammae sin. c. metast. pulm. Cholelithiasis. Spondylolisthesis V.L.V. Achylia gastrica. (DAT 256). Case No. 7 (♂ 41 yrs.). — Carcinoma ventriculi? Cirrhosis hepatis? Haematemesis. (DAT 64).

Various other diseases: Case No. 8 (♀ 35 yrs.). — Morbus Westergren. Hyperglobulinaemia. Insuff. hepatis. Icterus. Lues medicata seronegativa. (DAT 256). Case No. 9 (♀ 42 yrs.). — Ductus Botalli persistens.

Vit. valv. mitralis. Insuff. cordis. Cholecystopathia. Nephrolithiasis. Lues seropositiva. (DAT 128). Case No. 10 (♂ 71 yrs.). — Emphysema pulm. Bronchiectasiae. (DAT 128). Case No. 11 (♀ 65 yrs.). — Diabetes mellitus. Thrombophlebitis superficialis crur. dx. (DAT 63). Case No. 12 (♂ 62 yrs.). — Fractura pertrochanterica l. sin. (DAT 32). Case No. 13 (♀ 72 yrs.). — Myopathia cordis. Fibrillatio atriorum. Arteriosclerosis. Struma nodosa. Thyreotoxicosis. (DAT 256). Case No. 14 (♂ 63 yrs.). — Infarctus cordis? Insuff. cordis. Hypertensio arterialis. Cardiosclerosis. Anaemia sec. Diagnosis at autopsy: Aneurysma dissecans aortae. Nephritis chronica. (DAT 64). Case No. 15 (♀ 58 yrs.). — Struma nodosa. Compressio tracheae. (DAT 64). Case No 16 (♂ 75 yrs.). — St. p. fract. humeri sin. Arteriosclerosis. Aneurysma aortae abdominalis. Diabetes mellitus. (DAT 64). Case No. 17 (♀ 42 yrs.). — Dolorse abdominis. (DAT 256). Case No. 18 (♂ 33 yrs.). — Ulcus duodeni. Haematemesis. (DAT 64). Case No. 19 (♀ 51 yrs.). — Acrodermatitis atrophicans Herxheimer. (DAT 128). Case No. 20 (♀ 56 yrs.). — Bronchitis. (DAT 32).

Healthy persons: Case No. 21 (♀ 28 yrs.). — (DAT 64).

In cases No. 2, 3, 7, 8, 9, 10, 11, 15, 18, 19 and 20 the diagnosis was controlled by examination of the patient.

The following comments may be made on the »rheumatic anamnesis and status» in the above cases. In addition to cases No. 1 and 2, four patients had previously experienced one or more obvious attacks of rheumatic fever (cases No. 7, 8, 9 and 14) and one had manifested joint symptoms which may have been caused by rheumatic fever (case No. 18). No subjective or objective symptoms in the joints were seen in a single of these cases at the time of sample taking. Two other patients had previously had joint symptoms which may have been due to rheumatoid arthritis (cases No. 10 and 20). The latter had a distinct tendency to fusiformity in some fingers but no signs of active inflammation in the joints. Indefinite pains in the joints had been experienced earlier by five patients (cases No. 3, 11, 15, 16 and 21), two of whom (cases No. 11 and 16) had at the time of examination subjective joint sensations but no objective joint symptoms. In addition to case No. 3, two other patients reported ischiadic symptoms (cases No. 8 and 15).

Based on the above results it appears probable that the »positive» Waaler-Rose test can be accorded some value in the diagnosis of rheumatoid arthritis as far as the specificity of the occurrence of this reaction is concerned and provided that a sufficiently high DAT value is taken as the dividing line between the »positive» and the »negative» test results. However, the value of the test is restricted in the first place by the fact that a »positive» result was obtained in 61 per cent only of clinically typical cases of rheumatoid arthritis. It is further to be noted that a »positive» result was also obtained in one of the 18 cases of osteoarthritis (No. 4) and in two of the 65 cases in the rheumatic fever group. The patient with osteoarthritis had been under treatment in the surgical department, where a discission of the left obturator nerve had been performed. It was not possible to

control the diagnosis as the patient had left the hospital. One of the patients in the rheumatic fever group (case No. 2) had during 12 years suffered numerous attacks of rheumatic fever and for the past 4 years her condition had been continuously poor, with cardiac symptoms as the dominating feature in the picture, the sedimentation rate maintained a high level, and mild fever and marked anemia were coincidently present. Symptoms in the joints, on the other hand, were limited to indefinite sensations without objective findings. The other case of rheumatic fever (case No. 1) was listed in the hospital record as rheumatic fever with typical symptoms, which had set in after acute hepatitis. It is not possible to state whether or not the hepatitis in this case influenced the »positive» Waaler-Rose test result but it is interesting to note that out of the 21 »nonspecific» cases which gave a »positive» Waaler-Rose test without findings of rheumatoid arthritis, the diagnosis in 7 cases pointed to the possible presence of a hepatic lesion (cases No. 1, 5, 6, 7, 8, 9 and 19). The »nonspecifically positive» Waaler-Rose test results obtained in other cases than No. 1, 2 and 4 may be of minor practical significance in consideration of the fact that it rarely is difficult to differentiate between these disease groups and rheumatoid arthritis.

C. Correlation of the Waaler-Rose Phenomenon to the Duration of the Disease and the Sedimentation Rate in Cases of Rheumatoid Arthritis

A classification of 123 cases of rheumatoid arthritis was made into four groups according to the length of the anamnesis, as shown in table 3, and the number of »positive» cases (DAT $\geqq$ 32) was calculated in each group.

TABLE 3

CORRELATION OF THE DAT TO THE DURATION OF THE DISEASE IN 123 CASES OF RHEUMATOID ARTHRITIS

Length of anamnesis	No. of cases	DAT $\geqq$ 32	
		Cases	%
<2 yrs.	20	13	65
2–4 yrs.	40	19	47
5–9 yrs.	30	14	47
10–30 yrs.	33	16	48
	123	62	50

DAT = Differential agglutination titer

It will be noted from table 3 that the percentage of »positive» cases was higher in the group with an anamnesis of less than 2 years (65 per cent) than in the other three groups, in which the duration of the anamnesis was over two years (47, 47 and 48 per cent). However, the difference cannot be regarded as sufficiently significant to justify the drawing of general conclusions, particularly when the small size of the groups is taken into consideration. In order that the Waaler-Rose test would form a valuable aid in establishing the diagnosis in rheumatoid arthritis it would be of utmost importance that a »positive» reaction could be obtained already at an early stage of the disease, when the difficulty of diagnosis is by far the greatest. The series studied contained only 16 cases with an anamnesis of less than one year. Seven of these gave a »positive» Waaler-Rose test. The duration of the anamnesis was under six months in two cases only; one of these gave a DAT value of 4 and the other of 1024 (anamnesis 2 months).

A total of 138 cases of rheumatoid arthritis were classified into three groups according to the sedimentation rate, and the number of »positive» cases in each group was calculated.

TABLE 4

CORRELATION OF THE DAT TO THE SEDIMENTATION RATE IN 138 CASES OF RHEUMATOID ARTHRITIS

S. R.	No. of cases	DAT $\geqq$ 32	
		Cases	%
$\leqq$ 20	31	11	35
21—30	24	11	46
$\geqq$ 31	83	48	58
	138	70	51

DAT = Differential agglutination titer

Table 4 indicates that the percentage of »positive» reactions in each group was the greater the higher the sedimentation rate. No consistent correlation could nevertheless be seen in individual cases between the sedimentation rate and the DAT. In many cases with a very high sedimentation rate the DAT was under 16, and, on the contrary, the latter was sometimes very high with patients with a normal sedimentation rate.

Summary

The Waaler-Rose test was carried out with a total of 1291 human sera. When, in conformity with the practice of earlier investigators, the dividing line between »positive» and »negative» Waaler-Rose tests was placed at the DAT value of 16, a »positive» result was obtained not only with rheumatoid arthritis sera but also with numerous sera in the control groups. A DAT value of 32 proved more feasible as the borderline and on this basis the incidence of »positive» Waaler-Rose test values was as follows: In 46 per cent of 221 cases with a hospital diagnosis of rheumatoid arthritis or polyarthritis chronica (primaria) and in 61 per cent of 92 controlled cases of typical rheumatoid arthritis, but in 3 per cent only of 65 cases of rheumatic fever, 2 per cent of 886 cases of other diseases, and 1 per cent of 98 normal control cases.

Patients with rheumatoid arthritis gave a »positive» Waaler-Rose test value more frequently in cases with a duration of less than two years than in those with a longer anamnesis. However, the difference was not significant.

A »positive» Waaler-Rose test value was more rarely obtained in cases of rheumatoid arthritis with a slightly elevated sedimentation rate than in cases with a markedly elevated rate. In individual cases, however, no consistent correlation could be seen between the DAT value and the sedimentation rate.

CHAPTER IV

STUDIES ON THE NATURE AND MODE OF ACTION OF THE SERUM FACTOR RESPONSIBLE FOR THE WAALER-ROSE PHENOMENON

The red cells of sheep sensitized with rabbit anti-sheep cell amboceptor are used in the Waaler-Rose test. In the tests decribed in this chapter an attempt was made to elucidate (A) whether the serum factor which is responsible for the Waaler-Rose phenomenon acts also when a) other red cells and b) other sensitizing sera are used, and (B) whether the serum factor which is responsible for the Waaler-Rose phenomenon can be absorbed with nonsensitized or sensitized sheep red cells.

A. Agglutination Tests with Different Red Cells and Sensitizing Sera

A total of 12 different rheumatoid arthritis sera and 5 different normal sera was used. The technique was similar to that in the Waaler-Rose test (cf. p. 24) except that the final dilution series of the serum under examination was 1 : 2, 1 : 4, 1 : 8 and so on and that the human cells used in the tests were drawn into a citrate solution [1] (8 parts whole blood to 1 part citrate solution). The sensitized red cells used in the tests were the following:

1. a) Guinea pig cells, b) chicken cells, and c) human 0 cells, each sensitized with their homologous rabbit amboceptor;

2. Sheep cells sensitized with the homologous guinea pig amboceptor;

---

[1] Sodium citrate 2.67
Glucose 2.2
Citric acid 0.9
Aq. dest. . ..... ad 100.0

3. Sheep, guinea pig, rabbit and human 0 cells sensitized with the normal sera of these animals and man in all possible combinations;

4. Sheep cells sensitized with the serum of a patient with infectious mononucleosis;

5. Rhesus positive (0 Rh+) cells sensitized with the serum of a Rhesus immunized (0 Rh—) pregnant woman;

6. Human B cells sensitized with the serum of a person of blood group A.

The rabbit anti-guinea pig and anti-O cell amboceptor was produced by injecting intravenously into rabbits at intervals of 5 days 0.5, 1.0 and 2.0 ml. of a 5 per cent red cell suspension. The guinea pig anti-sheep cell amboceptor was obtained by intraperitoneal injection into guinea pigs at 4-day intervals of 0.5, 0.75, 1.0, 1.5, 2.0, 2.5 and 3.0 ml. of a 5 per cent sheep red cell suspension. The blood was taken from the guinea pigs and rabbits by heart puncture and the sera were inactivated. The rabbit anti-chicken cell amboceptor, which was obtained from the serum stock in the laboratory, had been produced by intravenously injecting chicken cell suspension into rabbits at intervals of 4 days in doses of 0.5, 1.0, 1.0, 1.0 and 1.0 ml.

Results

The main points in the results are shown in tables 5 and 6.

It should be pointed out in this connection that in the agglutination tests with sheep cells sensitized with normal rabbit serum the sera of three different rabbits were used as sensitizer. The negative results given by one of these sera are shown in table 6. Analogous results were obtained with the second serum, whereas the third rabbit serum, in tests carried out with 3 rheumatoid arthritis sera and 3 normal human sera and controlled several times, had an entirely analogous sensitizing action on sheep cells as had the homologous rabbit anti-sheep cell amboceptor in the Waaler-Rose test. Although the agglutination titer of the serum of this rabbit for nonsensitized sheep cells was only 1 : 8, the possibility must be considered that this was a former amboceptor rabbit which by error had been placed among the unimmunized animals.

The results in tables 5 and 6 would seem to indicate that the serum factor responsible for the Waaler-Rose phenomenon can act also when sheep cells are substituted by guinea pig, chicken or human 0 cells sensitized with their homologous rabbit amboceptors, and when sheep cells sensitized with the homologous guinea pig amboceptor instead of the homologous rabbit amboceptor are employed, even though all the guinea pig amboceptors did not give equally definite results. The factor had no effect on the agglutination

TABLE 5

AGGLUTINATION TESTS WITH 10 RHEUMATOID ARTHRITIS SERA AND 5 SERA OF HEALTHY PERSONS. ANTIGENS USED: NONSENSITIZED RED CELLS AND RED CELLS SENSITIZED WITH DIFFERENT SERA

Group	Serum	Dg.	Red cells	Sensitizing serum (1/2 x MAD)	Aggl. titer for NC	Aggl. titer for SC	DAT	DAT in Waaler-Rose test
1.a)	J.Y.	R	Guinea pig	Rabbit anti-guinea pig cell amboceptor (aggl.titer 1:600)	16	1024	64	256
	H.S.	R	» »	»	8	2048	256	256
	S.M.	R	» »	»	64	16384	256	512
	O.W.	H	» »	»	16	64	4	4
1.b)	A.N.	R	Chicken	Rabbit anti-chicken cell amboceptor (aggl. titer 1:8000)	32	2048	64	2048
	L.S.	R	»	»	16	16	1	2
	O.W.	H	»	»	8	16	2	4
1.c)	A.N.	R	Human O	Rabbit anti-human O cell amboceptor (aggl. titer 1:1000)	0	8192	>4096	2048
	E.K.	R	»	»	0	64	>32	32
	H.S.	R	»	»	0	1024	>512	256
	O.W.	H	»	»	0	4	>2	4
	M.I.	H	»	»	0	8	>4	2
2.	A.N.	R	Sheep	Guinea pig anti-sheep cell amboceptor No. 116 (aggl. titer 1:128)	32	16384	512	2048
	I.P.	H	»	»	32	32	1	2
	A.N.	R	»	Guinea pig anti-sheep cell amboceptor No. 117 (aggl. titer 1:512)	32	128	4	2048
	I.P.	H	»	»	32	32	1	2
	S.M.	R	»	Guinea pig anti-sheep cell amboceptor No. 333 (aggl. titer 1:400)	32	256	8	256
	S.N.	H	»	»	16	64	4	4
	A.N.	R	»	Guinea pig anti-sheep cell amboceptor No. 334 (aggl. titer 1:400)	32	1024	32	256
	N.L.	R	»	»	32	2048	64	32
	O.S.	R	»	»	16	512	32	128
	A.T.	R	»	»	64	128	2	4
	K.R.	H	»	»	16	16	1	4
4.	H.S.	R	»	Infectious mononucleosis serum (aggl. titer 1:256)	16	16	1	512
	A.V.	R	»	»	32	64	2	512
	O.W.	H	»	»	32	32	1	4

(Continued)

Group	Serum	Dg.	Red cells	Sensitizing serum (1/2 × MAD)	Aggl. titer for NC	Aggl. titer for SC	DAT	DAT in Waaler-Rose test
5.	A.N.	R	Human O Rh+	Serum of a Rh immunized pregnant woman (ORh−) (aggl. titer 1:400)	0	0	?	2048
	L.S.	R	»	»	0	0	?	2
6.	A.N.	R	Human B	Human A serum (aggl. titer 1:256)	64	64	1	2048
	O.W.	H	»	»	256	256	1	2

0 = No agglutination in serum dilution ≧ 1:2
NC = Nonsensitized red cells R = Rheumatoid arthritis MAD = Minimum agglutinating dose
SC = Sensitized red cells H = Healthy DAT = Differential agglutination titer

TABLE 6

AGGLUTINATION TITERS OF A RHEUMATOID ARTHRITIS SERUM (DAT IN WAALER-ROSE TEST = 512) AND THE SERUM OF A HEALTHY PERSON (DAT IN WAALER-ROSE TEST = 4) FOR NONSENSITIZED RED CELLS AND RED CELLS SENSITIZED WITH 1/2 x MAD OF ANIMAL AND HUMAN NORMAL SERA

	Rheumatoid arthritis serum (H. S.)					Serum of healthy person (O. W.)				
	Nonsensitized	Sensitized with sheep serum	Sensitized with rabbit serum	Sensitized with guinea pig serum	Sensitized with human O serum	Nonsensitized	Sensitized with sheep serum	Sensitized with rabbit serum	Sensitized with guinea pig serum	Sensitized with human O serum
Sheep cells ...	16		16	4	0	8		4	8	16
Rabbit cells ...	8	8		16	8	128	128		128	128
Guinea pig cells	4	4	4		4	32	32	8		64
Human O cells	0	0	0	0		0	0	0	0	

0 = No agglutination in serum dilution ≧ 1:2

Agglutination titer of sheep serum for » rabbit cells 1:32
» » » » » » guinea pig » 1:8
» » » » » » human O » 1:16
» » » guinea pig » » sheep » 1:2
» » » » » » » rabbit » 1:16
» » » » » » » human O » 1:4
» » » human O » » sheep » 1:8
» » » » » » » rabbit » 1:32
» » » » » » » guinea pig » 1:16
» » » rabbit » » sheep » 1:8
» » » » » » guinea pig » 1:2
» » » » » » human O » 1:16

of sheep cells sensitized with the serum of a patient with infectious mononucleosis, Rh positive cells sensitized with the serum of a Rh negative immunized pregnant woman, nor sheep, guinea pig, rabbit or human cells sensitized in all possible combinations with the normal sera of these animals and man. Nonsensitized cells were not agglutinated by rheumatoid arthritis sera more strongly than by normal human sera.

The action of the factor in the rheumatoid arthritis sera did not, accordingly, seem to be limited to one species of red cells or to one species of sensitizer sera. On the other hand, the sphere of action of the factor seemed to be limited in so far that not all the sensitized red cell systems but mostly the red cells sensitized with the homologous animal immune amboceptors were included.

The results obtained in these tests seemed to agree with W a a l e r ' s (54) explanation of the capacity of the rheumatoid arthritis sera to agglutinate sheep red cells sensitized with the homologous rabbit amboceptor. In W a a l e r ' s opinion this is the action of an »agglutination activating factor» which is characteristic of rheumatoid arthritis sera. This factor activates the agglutination between the sheep cells and the sensitizer-amboceptor so that agglutination occurs in high dilutions in which the amboceptor without the presence of the rheumatoid arthritis serum is ineffective. As also the results obtained by other investigators do not seem to conflict with W a a l e r's explanation, the concept »agglutination activating factor» (AAF) is hereafter used in this study to designate the serum factor which is the cause of the especially great capacity of certain human sera to agglutinate sensitized red cells.

Agglutination Tests with the Subject's Own Red Cells

To bring out the action of the AAF under conditions where the heterophilic hemagglutinins are eliminated as completely as possible, tests were made to find out whether rheumatoid arthritis sera are capable of agglutinating the subject's own red cells sensitized with the homologous rabbit amboceptor, and also whether any difference can be found in this respect between rheumatoid arthritis sera and normal human sera.

The sera and red cells of two patients with rheumatoid arthritis and two healthy persons were used in these tests. The technique

employed was the same as in the tests described above (cf. p. 37). The homologous rabbit amboceptors for sensitization of the cells were produced by immunizing rabbits as follows:

Amboceptor No. 1: Rabbit No. 1 was immunized with the cells of rheumatoid arthritis patient S. M. of blood group A;

Amboceptor No. 2: Rabbit No. 2 was immunized with the cells of rheumatoid arthritis patient I. H. of blood group O;

Amboceptor No. 3: Rabbit No. 3 was immunized with the cells of O. W., a healthy person of blood group O;

Amboceptor No. 4: Rabbit No. 4 was immunized with the cells of H. N., a healthy person of blood group A.

Sensitization of Red Cells. — In each case the cells were sensitized with the amboceptor produced with the same person's red cells. Thus S. M.'s cells were sensitized with amboceptor No. 1, those of I.H. with amboceptor No. 2, and so on.

The results obtained are shown in table 7.

TABLE 7

AGGLUTINATION TESTS WITH 2 RHEUMATOID ARTHRITIS SERA AND THE SERA OF 2 HEALTHY PERSONS. ANTIGENS USED: SUBJECT'S OWN RED CELLS, NONSENSITIZED AND SENSITIZED WITH AMBOCEPTOR

		Dilution of serum											
		2	4	8	16	32	64	128	256	512	1024	2048	4096
Rheumatoid arthritis serum (S. M.)	NC	−											
	SC	+++	++	++	++	++	++	++	++	+	+	−	−
Rheumatoid arthritis serum (I. H.)	NC	−											
	SC	++	++	++	++	++	+	+	+	+	−	−	−
Serum of healthy person (O. W.)	NC	−											
	SC	+	−	−	−	−	−	−	−	−	−	−	−
Serum of healthy person (H. N.)	NC	−											
	SC	+	+	+	−	−	−	−	−	−	−	−	−

NC = Subject's own nonsensitized red cells
SC = Subject's own red cells sensitized with 1/2 × MAD (minimum agglutinating dose) of amboceptor

It will be seem from table 7 that in all the four cases the investigated serum agglutinated the subject's own cells sensitized with rabbit amboceptor prepared with these cells. In both cases of rheumatoid arthritis this agglutination extended up to considerably higher serum dilutions (S. M. *ad* 1:1024, I. H. *ad* 1:512) than in the case of the healthy persons (O. W. *ad* 1:2, H. N. *ad* 1:8). The amboceptors alone in the dose used (1/2 × MAD) did not produce agglutination without the presence of the subject's serum, and, on the other hand, the subjects' sera did not agglutinate their own cells without the presence of amboceptor.

In further agglutination tests with the serum of the rheumatoid arthritis patient S. M. and the sensitized cells of the healthy person O. W., analogous results were obtained as with S. M.'s own cells. Further it appeared to be indifferent which of the four amboceptors (No. 1—4) was used as cell sensitizer.

Parallel with the above tests, the Waaler-Rose test also was carried out with the sera of the same four persons (table 8).

TABLE 8

UTINATION TESTS WITH 2 RHEUMATOID ARTHRITIS SERA AND THE SERA OF 2 HEALTHY PERSONS. ANTIGENS USED: SHEEP RED CELLS, NONSENSITIZED AND SENSITIZED WITH AMBOCEPTOR

		Dilution of serum										Saline control
		16	32	64	128	256	512	1024	2048	4096	8192	
eumatoid arthritis serum (S. M.)	NC	+++	+	−	−	−	−	−	−	−	−	
	SC	++++	++++	++++	+++	+++	+++	+++	++	+	−	−
eumatoid arthritis serum (I. H.)	NC	+++	++	−	−	−	−	−	−	−	−	
	SC	+++	+++	+++	+++	++	++	+	−	−	−	−
um of healthy person (O. W.)	NC	++	−	−	−	−	−	−	−	−	−	
	SC	+++	++	−	−	−	−	−	−	−	−	−
um of healthy person (H. N.)	NC	+++	++	−	−	−	−	−	−	−	−	
	SC	++++	+++	+++	+	−	−	−	−	−	−	−

= Nonsensitized sheep cells
= Sheep cells sensitized with 1/2 × MAD (minimum agglutinating dose) of amboceptor

Table 8 shows that the two rheumatoid arthritis sera agglutinated sensitized sheep cells in considerably higher serum dilutions than the sera of the healthy persons (S. M. *ad* 1:4096, I. H. *ad* 1:1024, O. W. *ad* 1:32, H. N. *ad* 1:128). Differing from the results obtained with the subjects' own nonsensitized cells, agglutination occurred in these tests with nonsensitized sheep cells as well. However, this agglutination did not in a single case extend beyond serum dilution 1:32 and no significant difference was seen between the sera of patients with rheumatoid arthritis and the sera of healthy persons.

The agglutination of the patient's own sensitized red cells in rheumatoid arthritis serum can by no means be due to the action of heterophilic hemagglutinins. Apparently this agglutination, and also the agglutination of other sensitized red cells in rheumatoid arthritis sera in cases in which an analogy with the Waaler-Rose phenomenon was seen, is produced by the same agglutination activating factor.

B. Absorption Tests with Nonsensitized and Sensitized Red Cells of Sheep

I. Absorption Tests to Remove the AAF from the Serum

A total of 7 different rheumatoid arthritis sera and 1 normal serum was used and absorption tests were made with sheep cells sensitized with the homologous rabbit anti-sheep cell amboceptor and with nonsensitized sheep cells.

Technique

Absorption Method A. — Equal parts of packed cells and serum dilution 1:4 in 0.9 per cent saline solution were mixed, kept in a water-bath at + 37° C. for two hours and carefully shaken every 10 minutes. The cells were separated by centrifugation.

Absorption Method B. — A mixture was made of α ml. of the serum dilution 1:10 in 0.9 per cent saline solution and $1\,1/2 \times \alpha$ ml. of packed cells, and kept overnight in the refrigerator at + 4° C. The cells were separated by centrifugation.

Sensitized Sheep Cells. — A 2 per cent suspension in saline solution was made of normal sheep cells which had been washed three times in large amounts of saline. An equal volume of amboceptor dilution

was added, the dose of amboceptor varying in the different tests. The suspension was kept in a water-bath at + 37° C. for two hours and carefully shaken every 10 minutes. The cells were separated by centrifugation and washed three times in large amounts of saline. In the absorption tests according to method B this centrifugation was made in a refrigerated centrifuge at a temperature of + 1— + 3° C. for 6 minutes at 1000—2000 RPM.

The technique used for sensitization was controlled as follows: A dose of 1/2 × MAD of amboceptor was used for sensitization of the cells. After centrifugation the supernatant fluid was retained and used for 1 per cent suspension of nonsensitized sheep cells. The Waaler-Rose test was carried out with a known rheumatoid arthritis serum, using this suspension instead of the cells sensitized in the usual manner. The DAT, which in the Waaler-Rose test was 4096, was now 2. The amboceptor serum, accordingly, had lost the sensitizing capacity which acts in the Waaler-Rose test. — The Waaler-Rose test was also performed with the same rheumatoid arthritis serum, substituting the sheep cells sensitized in the usual manner by cells which, after sensitization according to the above technique, had been washed three times and suspended in saline to form a 1 per cent suspension. The DAT now obtained was 4096. The sensitizing capacity of the amboceptor serum which acts in the Waaler-Rose test had accordingly become adsorbed to the sheep cells and remained so regardless of the washing.

TABLE 9

AGGLUTINATION TESTS WITH 4 RHEUMATOID ARTHRITIS SERA AND THE SERUM OF A HEALTHY PERSON BEFORE AND AFTER ABSORPTION BY METHOD A. ANTIGENS USED: SHEEP RED CELLS, NONSENSITIZED AND SENSITIZED WITH AMBOCEPTOR

Serum	Dg.	Titer of serum for non-sensisitized sheep cells			Titer of serum for sensitized sheep cells			DAT		
		Non-absorbed	Absorbed with NC	Absorbed with SC	Non-absorbed	Absorbed with NC	Absorbed with SC	Before absorption	After adsorption with NC	After adsorption with SC
S.M.	R	64	0	0	8192	8192	8192	128	>1024	>1024
G.S.	R	32	0	0	8192	8192	2048	256	>1024	>256
X.N.	R	16	0	0	4096	4096	1024	256	>512	>128
H.S.	R	16	0	0	1024	1024	512	64	>128	>64
H.N.	H	64	0	0	128	64	16	2	>8	>2

0 = No agglutination in serum dilution ≧ 1:8
NC = Nonsensitized sheep cells
SC = Sheep cells sensitized with 1/2 × MAD (minimum agglutinating dose) of amboceptor
R = Rheumatoid arthritis
H = Healthy
DAT = Differential agglutination titer

The agglutination titers of the sera for nonsensitized and sensitized sheep cells were determined by the Waaler-Rose test technique before and after absorption.

Absorption tests according to *method A* were made with 4 rheumatoid arthritis sera and 1 normal serum. For sensitization of the sheep cells 1/2 × MAD of amboceptor was used.

Results

It will be seen from table 9 that absorption of sera both with sensitized and nonsensitized sheep cells removed the capacity to agglutinate nonsensitized sheep cells in all cases (no agglutination in serum dilution $\geq$ 1:8). Absorption with nonsensitized sheep cells had no appreciable effect on the capacity to agglutinate sensitized sheep cells. On the other hand, it seemed probable that absorption with sensitized cells reduced this capacity to some degree but the reduction in the titers was so small that no definite conclusions are justified.

As it seemed possible that an improvement of the absorption technique might give more definite results, a series of tests was made with one rheumatoid arthritis serum and sheep cells sensitized with 1/2 × MAD of amboceptor in order to find the serum dilution and amount of sensitized cells which are optimal for absorption. Serum dilutions of 1 : 4, 1 : 8 and 1 : 16 (2 ml. used in each test) were absorbed with 1.0, 0.5 and 0.25 ml. of sensitized and packed cells in all possible combinations of serum dilution and amount of cells. The best combination for the absorption was found to be a serum dilution of 1 : 16 and 1 ml. of sensitized and packed cells. In this manner the agglutination titer of rheumatoid arthritis serum for sensitized cells could be reduced to one-fourth and in all the other combinations to one-half at the most. No decisive change in the absorption results was therefore obtained by changing the quantitative relation between the serum to be absorbed and the red cells.

The next problem to be studied was the possible effect on absorption of sensitization of sheep cells with a big dose of amboceptor. One rheumatoid arthritis serum (1.5 ml. of a 1 : 16 dilution) was absorbed with sheep cells (4.5 ml. of packed cells) sensitized with 5 × MAD of amboceptor. After absorption the serum was dark red in colour due to partial hemolysis of the cells. The agglutination titer for sheep cells sensitized with 1/2 × MAD was increased 4-fold, probably due to some amboceptor having become released in connection with the cell hemolysis.

A further absorption test was made with the same rheumatoid arthritis serum as follows: A mixture was made of 2 ml. of the serum dilution 1 : 16 and 0.043 ml. of undiluted amboceptor (corresponding to a dose of amboceptor required for sensitizing 6 ml. of packed sheep cells with 5 × MAD of amboceptor). The mixture was put into a water-bath of +37°C. for one hour, after which 6 ml. of nonsensitized

TABLE 10

AGGLUTINATION TESTS WITH A RHEUMATOID ARTHRITIS SERUM BEFORE AND AFTER ABSORPTION BY METHOD B. ANTIGEN USED: SHEEP RED CELLS SENSITIZED WITH 1/2 × MAD OF AMBOCEPTOR

Rheumatoid arthritis serum (A.V.)	Dilution of serum									
	40	80	160	320	640	1280	2560	5120	10240	20480
Nonabsorbed	+++	+++	++	++	++	(++)	(++)	+	(+)	−
Absorbed with NC	+++	+++	++	++	++	+	+	(+)	−	−
Absorbed with SC sensitized with 1/2 × MAD	++	++	++	++	+	(+)	(+)	−	−	−
Absorbed with SC sensitized with 1 × MAD	++	++	++	(+)	−	−	−	−	−	−
Absorbed with SC sensitized with 5 × MAD	++	(+)	(+)	−	−	−	−	−	−	−

NC = Nonsensitized sheep cells
SC = Sensitized sheep cells
MAD = Minimum agglutinating dose

TABLE 11

AGGLUTINATION TITERS OF 3 RHEUMATOID ARTHRITIS SERA BEFORE AND AFTER ABSORPTION BY METHOD B, FOR SHEEP RED CELLS SENSITIZED WITH 1/2 × MAD OF AMBOCEPTOR

Rheumatoid arthritis serum	Non-absorbed	Absorbed with NC	Absorbed with SC sensitized with			
			1/2 × MAD	1 × MAD	2 × MAD	5 × MAD
A.V.	5120	2560	640	160		40
S.M.	10240				80	40
A.N. (I abs.)	32768	20480			640	
» (II abs.)		10240			0	

0 = No agglutination in serum dilution $\geqq$ 1:20
NC = Nonsensitized sheep cells
SC = Sensitized sheep cells
MAD = Minimum agglutinating dose

sheep cells were added. The mixture was held in the water-bath at +37°C. for another hour and the cells were then separated by centrifugating. The titer for sheep cells sensitized with 1/2 × MAD had increased 4-fold as compared with the corresponding titer of the rheumatoid arthritis serum prior to absorption.

Thus the AAF could not be removed from the sera by means of these absorption tests, all of which were carried out at + 37° C.

Absorption tests were made according to *method B* with 3 rheumatoid arthritis sera, using for the absorption both nonsensitized sheep cells and sheep cells sensitized with varying doses of amboceptor.

Results

Table 10 and 11 show that no significant reduction in the agglutination titers of the sera for sensitized cells was obtained by absorption with nonsensitized cells. On the other hand, absorption with sensitized cells definitely lowered the titer and this reduction was the greater the bigger the dose of amboceptor used for sensitization of the cells used for absorption. From the results obtained it seems probable that the AAF can be completely removed from the sera by absorption with sensitized sheep cells and that the absorption is the more effective the bigger the dose of amboceptor used for cell sensitization.

As some red coloring due to partial hemolysis of the cells was observed in the sera after absorption with highly sensitized cells it was considered necessary to control whether or not hemolysis might lower the agglutinating capacity of the sera for sensitized sheep cells. This control tests was performed as follows:

A 25 per cent suspension of normal sheep cells was made in 0.9 per cent saline solution, kept in the water-bath at +56°C. for two hours, centrifugated for 15 minutes, mixed, and centrifugated once more. This centrifugation and mixing was done four times, resulting in complete hemolysis of the cells. From the resultant clear, dark red fluid a hemolysis dilution series was made as follows: 0.5 ml. of saline solution was first measured into each tube; 0.5 ml. of the above mentioned fluid was added to the first tube, from which 0.5 ml. was then transferred to the second tube, and so on throughout the series of tubes. Into the first tube was then added 0.5 ml. of diluted (1 : 10) nonabsorbed rheumatoid arthritis serum, and dilution was continued through the entire tube series as above. Then 0.5 ml. of 1 per cent suspension of sheep cells sensitized with 1/2 × MAD of amboceptor was added to each tube. The tube series was kept in the water-bath at +37°C. for one hour and overnight in the refrigerator. The agglutination reading was then taken in the usual manner. It was found that the agglutination titer was as high (1 : 20480) as in a test carried out concurrently in which the rheumatoid arthritis serum had been diluted with saline solution in the usual way. It is therefore probable that hemolysis in itself does not reduce the capacity of rheumatoid arthritis sera to agglutinate sensitized sheep cells.

II. Absorption and Elution Tests to Isolate the AAF from Sheep Cells Used for Absorption

The tests described above indicated that the AAF can be absorbed from the sera by means of highly sensitized sheep cells. The following tests were performed in order to study whether the AAF adsorbed to the sensitized cells can be released and thus isolated. A total of 5 rheumatoid arthritis sera and 1 normal serum was used in these tests, and control tests were made using the same technique but substituting the serum by saline solution.

Technique

The sheep cells to be used for the absorption were sensitized with 2 × MAD of amboceptor, using the technique described above (cf. p. 44). Three ml. of undiluted serum from a patient with rheumatoid arthritis were absorbed with 1.5 ml. of sensitized and packed cells, keeping the mixture overnight in the refrigerator. The cells were separated by centrifugating and washed three times in 3 ml. of saline solution. The saline solutions used for washing (I, II and III) were retained. To release the AAF from the cells they were eluated by the Landsteiner method (27) in the following manner: The cells were mixed in a small centrifuge tube with a triple volume of saline solution, and the tube was then held in + 56° C. water for 5 minutes and carefully shaken all the time. Immediately thereupon the cells were quickly separated by centrifugating. This was done by placing the small centrifuge tube in a larger one containing + 56° C. water. After centrifugation the supernatant fluid was retained. A part of it was absorbed with twice the volume of nonsensitized sheep cells, the tube being kept in the water-bath at + 37° C. for one hour. This was done to remove the sheep cell agglutinins which may have been released from the cells during elution. — Changes in the agglutination titer for nonsensitized sheep cells and sheep cells sensitized with 1/2 × MAD of amboceptor were determined by the Waaler-Rose test technique in the various stages of this series of tests.

Similar absorption and elution tests were also made by substituting nonsensitized sheep cells for sensitized cells.

Results

It is seen from table 12 that the agglutinating capacity of the sera

TABLE 12

VGGLUTINATION TITERS FOR NONSENSITIZED SHEEP RED CELLS AND FOR SHEEP RED CELLS SENSITIZED WITH 1/2 × MAD OF AMBOCEPTOR IN ABSORPTION AND ELUTION TESTS WITH 5 RHEUMATOID ARTHRITIS SERA AND THE SERUM OF A HEALTHY PERSON. FOR ABSORPTION: SHEEP RED CELLS SENSITIZED WITH 2 × MAD OF AMBOCEPTOR

	R-serum (A.N.)			R-serum (A.V.)			R-serum (H.S.)			R-serum (G.S.)			R-serum (L.S.)			H-serum (O.W.)			Saline control		
	NC	SC	DAT	NC	SC	DAT	NC	SC	DAT	NC	SC	DAT	NC	SC	DAT	NC	SC	DAT	NC	SC	DAT
Nonabsorbed serum	32	32768	1024	8	16384	2048	4	32768	8192	16	16384	1024	32	128	4	16	128	8	0	0	?
Serum absorbed with SC sensitized with 2 × MAD	0	32768	> 16384	0	16384	> 8192	0	8192	> 4096	0	8192	> 4096	0	16	> 8	0	8	> 4	0	0	?
Saline from I washing of SC used for absorption ...	2	512	256				0	256	> 128	0	1024	> 512				0	16	> 8			
Saline from II washing of SC used for absorption ...	2	64	32				0	8	> 4	0	32	> 16				0	8	> 4			
Saline from III washing of SC used for absorption ...	0	8	> 4				0	4	> 2	0	8	> 4				0	4	> 2			
Supernatant fluid after elution of SC used for absorption	64	1024	16	8	256	32	16	128	8	16	1024	64	16	32	2	16	64	4	16	16	1
Supertanant fluid absorbed with NC	0	512	> 256	0	512	> 256	0	128	> 64	0	512	> 256	0	4	> 2	0	8	> 4	0	0	?

NC = Nonsensitized sheep cells
SC = Sensitized sheep cells
0 = No agglutination in dilution $\geqq$ 1:2
MAD = Minimum agglutinating dose
DAT = Differential agglutination titer
R-serum = Rheumatoid arthritis serum
H-serum = Serum of a healthy person

for nonsensitized sheep cells was removed in all cases by absorption with sensitized sheep cells (no agglutination in serum dilution $\geqq$ 1:2). The agglutination titer for sensitized sheep cells was reduced by 0—2 tubes in the case of sera with a high DAT and 3—4 tubes in the case of those with a low DAT. This is quite comprehensible, as the capacity of the sera with a high DAT (*i.e.* with a large amount of AAF) to agglutinate sensitized cells probably is mainly due to the AAF, and the small amounts of sensitized cells used were not capable of absorbing the latter. In the case of sera with a low DAT (*i.e.* with a small amount of AAF) this capacity may, in the first hand, be ascribed to the heterophilic sheep cell agglutinins in these sera, which were removed in the absorption process.

The I saline solutions used for washing the sensitized cells employed for absorption of the sera were in all cases capable of agglutinating sensitized cells (titers in cases of rheumatoid arthritis 1:512, 1:256, 1:1024, in the normal case 1:16). This was doubtlessly due to loose nonabsorbed AAF remaining among the cells at this stage. The titer for sensitized cells was lower in washing solutions II and III, being in the latter solution in each case $\leqq$ 1:8, which indicates that triple washing almost completely removed all loose nonabsorbed AAF from among the cells. If cells which have been washed in this manner are eluated with saline in a water-bath at + 56° C., the supernatant fluid from the elution causes again a stronger agglutination of sensitized cells (titers in cases of rheumatoid arthritis 1:1024, 1:256, 1:128, 1:1024, 1:32, in the normal case 1:64), but it causes agglutination of nonsensitized sheep cells to some extent as well (titers in cases of rheumatoid arthritis 1:64, 1:8, 1:16, 1:16, in the normal case 1:16). The probable explanation of this phenomenon is that the AAF is actually adsorbed specifically to sensitized cells and released again upon elution of the cells. At the same time some of the heterophilic sheep cell agglutinins and specific sheep cell agglutinins of the sensitizing amboceptor serum which have been adsorbed to the cells are released. When these sheep cell agglutinins have been finally removed by absorption with nonsensitized sheep cells, the agglutination titer of the post-absorption supernatant fluid for sensitized sheep cells reflects directly the concentration of the AAF which has been adsorbed from the serum to the sensitized sheep cells and again released during elution (titers in cases of rheumatoid arthritis 1:512, 1:512, 1:128, 1:512, 1:4, in the normal case 1:8).

DAT = Differential agglutination titer H-serum = Serum of a healthy person

TABLE 13

AGGLUTINATION TITERS FOR NONSENSITIZED SHEEP RED CELLS AND FOR SHEEP RED CELLS SENSITIZED WITH 1/2 × MAD OF AMBOCEPTOR IN ABSORPTION AND ELUTION TESTS WITH A RHEUMATOID ARTHRITIS SERUM AND THE SERUM OF A HEALTHY PERSON. FOR ABSORPTION: NONSENSITIZED SHEEP RED CELLS

	Rheumatoid arthritis serum (A.N.)			Serum of healthy person (O.W.)		
	NC	SC	DAT	NC	SC	DAT
Nonabsorbed serum	32	32768	1024	16	128	8
Serum absorbed with NC	0	32768	>16384	0	32	>16
Saline from I washing of NC used for absorption	16	2048	128	0	16	> 8
Saline from II washing of NC used for absorption	8	256	32	0	8	> 4
Saline from III washing of NC used for absorption	8	32	4	0	2	> 1
Supernatant fluid after elution of NC used for absorption	16	16	1	4	32	8
Supernatant fluid absorbed with NC	0	16	>8	0	2	>1

0 = No agglutination in dilution $\geqq$ 1:2

NC = Nonsensitized sheep cells
SC = Sensitized sheep cells
MAD = Minimum agglutinating dose
DAT = Differential agglutination titer

It will be noted that these titers are in correlation with the DAT values obtained with these sera in the Waaler-Rose test.

As a control, the corresponding absorption and elution tests were made with 1 rheumatoid arthritis serum and 1 normal serum, using nonsensitized sheep cells. As seen from table 13, the final supernatant fluid resulting from the last absorption with nonsensitized cells was also in this case able to agglutinate sensitized cells to some extent but this agglutination was so weak (titers 1:16 and 1:2) that there probably is a question here of a nonspecific adsorption of the AAF to the nonsensitized sheep cells.

Summary

The rheumatoid arthritis sera which were capable of producing a strong agglutination of sheep cells sensitized with the homologous rabbit amboceptor had a similar action on the following sensitized cells:

— guinea pig, chicken and human 0 cells sensitized with the homologous rabbit amboceptor;

— sheep cells sensitized with the homologous guinea pig amboceptor;

— the patient's own cells sensitized with the homologous rabbit amboceptor.

No analogous effect was seen on the following sensitized cells:

— sheep cells sensitized with the serum of a patient with infectious mononucleosis;

— Rh positive cells (0 Rh +) sensitized with the serum of a Rh immunized pregnant woman (0 Rh −);

— human B cells sensitized with the serum of a person of blood group A;

— sheep, guinea pig, rabbit and human 0 cells sensitized with the normal sera of these animals and man in all possible combinations.

The strong agglutinating capacity of the rheumatoid arthritis sera for red cells sensitized with the homologous amboceptor is probably to be accounted for by an agglutination activating factor (AAF), which is characteristic of these sera.

The AAF was not removable from the sera by means of absorption with nonsensitized or sensitized sheep cells at + 37° C.

The AAF was, however, removable from the sera by means of absorption with sensitized sheep cells at + 4° C. The absorption was the more effective the higher the sensitization of the cells used for absorption.

The AAF which had been adsorbed to the sensitized sheep cells was releasable by elution of the cells in a saline solution at + 56° C.

CHAPTER V

CORRELATION OF THE SERUM FACTOR RESPONSIBLE FOR THE WAALER-ROSE PHENOMENON TO THE CAPACITY OF RHEUMATOID ARTHRITIS SERA TO AGGLUTINATE HEMOLYTIC STREPTOCOCCI

The tests described in Chapter IV indicated that the strong agglutinating power of rheumatoid arthritis sera for sensitized red cells may be attributed to an agglutination activating factor (AAF) present in these sera. In the tests to be described in this chapter an attempt was made to discover whether the AAF also plays a role in the capacity of the rheumatoid arthritis sera to agglutinate hemolytic streptococci.

The working hypothesis was the following:

If the serum under examination is absorbed with hemolytic streptococci the streptococcal agglutinins will be removed. The agglutination activating factor will remain in the rheumatoid arthritis serum but in the absence of streptococcal agglutinins it does not produce agglutination. If a fresh agglutination test is made with absorbed serum using a streptococcal antigen which has been sensitized with a small amount of a known streptococcal immune serum, agglutination of the sensitized streptococci can be expected to occur in case the absorbed serum contains the agglutination activating factor.

Technique

The technique described by Thulin (52) was employed in all the agglutination and absorption tests with streptococci. The Waaler-Rose test was performed in the usual manner (cf. p. 24).

Preparation of the Streptococcal Antigen. — Strain: *Streptococcus haemolyticus* strain S.F. 130, belonging to group A, type 1. The same strain was used by Kalbak (26), among others, in routine examinations of streptococcal agglutinins.

Two different types of broth were employed, viz. broth I = neopeptone broth, and broth II = bactopeptone broth[1].

Broth I was inoculated with a liberal amount of strain S.F. 130 and incubated at +37°C. for 6 hours. Then 50 ml. of the incubated broth I was transferred to 150 ml. of broth II, which was incubated for 10—12 hours. This broth culture was used in the agglutination tests as antigen.

Agglutination Technique. — With 0.3 per cent saline solution a dilution series of 1 : 10, 1 : 20, 1 : 40 and so on was made from inactivated serum, placing 0.2 ml. of the dilution in each tube. An equal amount of antigen was added and the tubes were placed in the water-bath at +52°C. for two hours and then in the refrigerator overnight. The agglutination reading was taken in a dark room by viewing the tubes against a lamp while they were being shaken cautiously. The intensity of the agglutination was estimated according to Kalbak (26) as follows:

— = Diffuse specimen with no agglutination;
± = Weak, indefinite agglutination;
+ = Sediment broken up to fairly large coherent fragments; supernatant fluid clear;
++ = Sediment consists of large coherent fragments; supernatant fluid clear;
+++ = Sediment forms one large solid cake that is difficult to break by shaking.

Agglutination of intensity + or stronger is designated as the »positive test».

Absorption Technique. — The centrifugate of 1 liter of broth culture II, prepared as described above, was suspended in 2 ml. of a serum dilution 1 : 10, incubated at +37°C. for two hours and stored overnight in the refrigerator.

Production of the Streptococcal Immune Serum. — Strain S.F. 130 was inoculated into 50 ml. of broth II and incubated at +37°C. for 24 hours. Then 0.5 per cent of formalin was added and the vaccine so prepared was injected intravenously into a rabbit at intervals of 3 days in doses of 0.25—0.5—0.75—1.0—1.25—2.5—3.5 ml. Blood was drawn by heart puncture, and the serum was separated and inactivated.

The immune serum was titrated as described above for the agglutination test. Diverging from this technique the dilution series was made as follows: Dilutions

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[1] *Broth for Preparation of Streptococcal Antigen:*

750 g. chopped beef
1.5 l. tap water.

The mixture is left standing in refrigerator (+ 4° C) overnight. Then boiling for 15 min. Filtration through filter paper. Then addition of 5/n NaOH to pH 8.0. Boiling for 30 min.

I. *Neopeptone Broth:*

To 500 ml. broth, with pH 8.0, are added:

Neopeptone (Difco) ..... 5 g.
Na_2HPO_4, 12 H_2O ..... 2 g.

Boiling. Adjustment to pH 8.0.

Filtration through paper and distribution into flasks of 50 ml. Then autoclaving at 120° for 20 min.

II. *Bactopeptone Broth:*

To 1 liter broth, with pH 8.0, are added:

Bactopeptone (Difco) ... 10 g.
NaCl ..... 5 g.

Boiling. Adjustment to pH 8.0. Filtration through paper. Distribution into flasks of 150 ml. Autoclaving at 120° for 20 min. Then adjustment to pH 7.6—7.8.

were made from the immune serum in the ratio 1 : 20, 1 : 50 and 1 : 60, 10 ml. of each. Using 0.2 ml. of each of these three initial dilutions, series were made in powers of 2, diluting as described above. — The agglutination titer of the immune serum varied from 1 : 1600 to 1 : 2400 in the different titrations.

Experiments

A total of 10 different sera was used, representing the following groups:

I. Clinically typical cases of rheumatoid arthritis (4 cases);

II. A clinically uncertain borderline case between rheumatoid arthritis and rheumatic fever (1 case);

III. Cases with a probable etiology of streptococcal infection (3 cases);

IV. A healthy person (1 case);

V. A rabbit immunized with streptococci (1 case).

TABLE 14

DIAGNOSES, STREPTOCOCCAL AGGLUTINATION TITERS AND DAT VALUES OF 10 SERA

Group	Serum	Clinical diagnosis	Streptococcal agglutination titer	DAT in Waaler-Rose test
I	O.S.	Rheumatoid arthritis	1:320	2048
	S.M.	» »	1:640	1024
	A.R.	» »	1:320	512
	E.P.	» »	1:320	64
II	V.L.	Rheumatic fever? Rheumatoid arthritis?	1:640	512
III	O.R.	Abscessus peritonsillaris	+1:80±1:160	8
	E.S.	Nephritis acuta Tonsillitis chronica	+1:80±1:160	4
	A.S.	Carditis? Insufficientia cordis. Fibrillatio atriorun. Bronchitis ...	1:640	4
IV	O.W.	Healthy	0	2
V	Rabbit No.52	Immunized with *Streptococcus haemolyticus* strain S. F. 130	1:2560	1

DAT = Differential agglutination titer
0 = No agglutination in serum dilution $\geqq$ 1:20

Table 14 shows the agglutination titers of the sera for streptococci and the DAT values obtained in the Waaler-Rose test. Group III was to comprise cases with known streptococcal diseases but difficulties were experienced in obtaining such sera with a high agglutination titer for streptococci. More than 200 sera were examined. These were obtained from patients undergoing treatment under the diagnoses scarlatina, erysipelas, tonsillitis, angina tonsillaris, nephritis, etc., and from patients from whose pharyngeal samples *Streptococcus haemolyticus* had been cultured. Very few of these sera agglutinated streptococci, and most of them in titers $\leqq 1{:}40$. The reason for this may, of course, be that in very few of these cases the disease was caused by infection with type 1 streptococci. There was no difficulty, however, in finding rheumatoid arthritis sera with a strong streptococcal agglutination. Out of 83 rheumatoid arthritis sera 66 (79 per cent) had a streptococcal agglutination titer of $\geqq 1{:}20$, 52 (63 per cent) $\geqq 1{:}40$, 39 (47 per cent) $\geqq 1{:}80$, and 22 (26 per cent) $\geqq 1{:}160$. In the Waaler-Rose test 51 (61 per cent) of these 83 sera had a DAT value of $\geqq 16$, and 32 (38 per cent) of $\geqq 32$. In the cases in which the streptococcal agglutination was »positive» ($\geqq 1{:}20$) but the Waaler-Rose test »negative» (DAT < 32) only 5 had a streptococcal agglutination titer of $\geqq 1{:}80$. There were only 5 cases in which the Waaler-Rose test was »positive» (DAT $\geqq 32$) but the streptococcal agglutination »negative» (in 4 of these the streptococcal agglutination was $\pm$ in the dilution 1:20). It therefore seems as if the streptococcal agglutination in rheumatoid arthritis were »positive» more often than the Waaler-Rose test. There seems to exist a certain correlation between a »positive» Waaler-Rose test and a »positive» streptococcal agglutination in rheumatoid arthritis, although they do not always occur concurrently with the same sera.

Main test. — The sera under examination, with the exception of that of the healthy person (O. W.), were absorbed with streptococci. The following tests were carried out simultaneously with absorbed and nonabsorbed sera:

a) Determination of the agglutination titer of the serum for nonsensitized streptococci (table 15, points 1 and 5);

b) Determination of the agglutination titer of the serum for streptococci sensitized with varying doses of immune serum (table 15,

TABLE 15

AGGLUTINATION TESTS WITH ABSORBED AND NONABSORBED SERA. ANTIGENS USED: NONSENSITIZED STREPTOCOCCI AND STREPTOCOCCI SENSITIZED WITH STREPTOCOCCAL IMMUNE SERUM

	Dose of immune serum used for sensitization of streptococci	SERA									
		I				II	III			IV	V
		O.S.	S.M.	A.R.	E.P.	V.L.	O.R.	E.S.	A.S.	O.W.	Rabbit No. 52
Before absorption with streptococci	1) None	1:320	1:640	1:320	1:320	1:640	+1:80 ±1:160	+1:80 ±1:160	1:640	0	1:2560
	2) 1/2×MAD	1:320		+1:320 ±1:640	1:640	1:640			1:640	0	1:1280
	3) 1/4×MAD	1:320		1:320	1:320	1:640	1:80		+1:320 ±1:640	0	1:1280
	4) 1/8×MAD	1:320		1:320	1:320	1:640			1:640		1:1280
After absorption with streptococci	5) None	0	0	0	0	0	0	0	0		0
	6) 1/2×MAD	1:320	1:1280	+1:40 ±1:80	1:640	1:160	0	0	0		0
	7) 1/4×MAD	1:80	+1:160 ±1:320	1:40	+1:320 ±1:640	1:160	0	0	0		0
	8) 1/8×MAD	1:40	1:160	1:40	1:160	1:160		0	0		0
	9) 1/16×MAD	1:40		0	+1:40 ±1:80	1:80		0	0		0
	DAT in Waaler-Rose test	2048	1024	512	64	512	8	4	4	2	1

0 = No agglutination in serum dilution $\geqq$ 1:20

MAD = Minimum agglutinating dose DAT = Differential agglutination titer

points 2, 3, 4, 6, 7, 8 and 9). The streptococci were sensitized by adding to the streptococcal antigen prior to the test an amount of undiluted immune serum corresponding to the »agglutinating dose» desired.

In addition, the Waaler-Rose test was carried out with all the nonabsorbed sera.

The results of these tests are shown in table 15.

It is seen from table 15 that the nonabsorbed sera agglutinated the sensitized and nonsensitized streptococci almost equally (points 1—4). The case was different with the absorbed sera, as not a single one of these were capable of agglutinating nonsensitized streptococci in the lowest serum dilution used, *i.e.* 1:20 (point 5). Sensitized streptococci, on the other hand, were agglutinated by the absorbed sera in groups I and II and the agglutination titers were the higher the bigger the dose of immune serum used for sensitization of the streptococci (points 6, 7, 8 and 9). The absorbed sera in groups III and V did not agglutinate sensitized streptococci (points 6, 7, 8 and 9). It is also noted that all the sera which agglutinated sensitized streptococci after absorption gave a strongly »positive» Waaler-Rose test, and those which did not agglutinate sensitized streptococci after absorption gave a »negative» Waaler-Rose test.

In the preceding tests with sensitized streptococci equally large doses of sensitizing immune serum were placed in all the tubes in the dilution series. This does not fully correspond to the conditions in the agglutination tests with nonsensitized streptococci and nonabsorbed sera. According to the working hypothesis the agglutination of streptococci by nonabsorbed rheumatoid arthritis sera is due to the circumstance that the streptococcal agglutinins which are generally present in the human serum sensitize the streptococcal antigen, which then becomes agglutinated in the presence of the AAF. When a dilution series is made with nonabsorbed serum the concentration of the streptococcal agglutinins is diluted in the same way. In order to prepare similar test conditions when using absorbed sera and sensitized streptococci, additional control agglutination tests were made with absorbed sera by placing 1/2 × MAD of the sensitizing immune serum in the first tube, 1/4 × MAD in the second, and so on.

These tests were carried out as follows: 1 × MAD of immune

MAD = Minimum agglutinating dose
DAT = Differential agglutination titer

serum in 0.2 ml. of 0.3 per cent saline was measured into the first tube and 0.2 ml. of saline into the other tubes. To the first tube was added 0.2 ml. of absorbed serum dilution 1:10, 0.2 ml. was transferred from the first tube to the second tube, and this was continued through the series of tubes. A similar amount, 0.2 ml., of nonsensitized streptococcal antigen was added to each tube. The balance of the test was performed as described above (cf. p. 55). A simultaneous control series was made with immune serum only, without the presence of the serum under examination.

TABLE 16

AGGLUTINATION TESTS WITH SERA ABSORBED WITH STREPTOCOCCI. ANTIGEN USED: STREPTOCOCCI SENSITIZED WITH DECREASING DOSES OF IMMUNE SERUM

Group	Serum	Sensitizing dose of immune serum: MAD/2	MAD/4	MAD/8	MAD/16	MAD/32
		Dilution of serum under test: 40	80	160	320	640
I	O.S.	++	+	−	−	−
	A.R.	+	±	−	−	−
	E.P.	+++	++	+	−	−
II	V.L.	++	++	+	−	−
III	O.R.	−	−	−	−	−
	E.S.	−	−	−	−	−
	A.S.	−	−	−	−	−
V	Rabbit No. 52	−	−	−	−	−
	Saline control	−	−	−	−	−

MAD = Minimum agglutinating dose

Table 16 shows that the agglutination results were analogous to those in the main test.

Further absorption tests were carried out as follows: Two rheumatoid arthritis sera (DAT 128 and 1024) were absorbed by method B (cf. p. 44) with sheep cells sensitized with 2 × MAD of the homologous rabbit anti-sheep cell amboceptor. After this the

sera did not agglutinate sensitized or nonsensitized sheep cells in the Waaler-Rose test. However, the agglutination titers of the sera against streptococci (prior to absorption 1:320 and 1:80) had increased to 1:2848 and 1:16384 respectively. As after the absorption process the sera were somewhat reddish due to hemolysis, a hemolysis control test was performed. Nonsensitized sheep cells were added to a 0.3 per cent saline solution in such an amount that hemolysis of the cells gave it a reddish tinge which macroscopically estimated was slightly darker than that of the above mentioned absorbed sera. A hemolysis dilution series was made from this fluid using 0.3 per cent saline, streptococcal antigen was added to the tubes, and the test was continued as before (cf. p. 55). It was found that already mere hemolysis, without the presence of the rheumatoid arthritis serum, produced definite streptococcal agglutination up to the tenth tube (corresponding to serum dilution 1:16384). — One rheumatoid arthritis serum was also absorbed with streptococci, using the same serum as above with a DAT value of 128 and a streptococcal agglutination titer of 1:320. After absorption the serum did not agglutinate streptococci. The agglutination titer for sensitized sheep cells was reduced to one-fourth (from 1:4096 to 512).

These tests accordingly showed that rheumatoid arthritis sera absorbed with streptococci are capable of agglutinating streptococci sensitized with the homologous rabbit immune serum and that the agglutination titer is directly proportionate to the dose of immune serum used for sensitization. This phenomenon cannot be ascribed to the streptococcal agglutinins which regardless of the absorption may have remained in the serum nor to the agglutinins in the immune serum used for sensitization of the antigen. Even together these agglutinins would be too weak to produce agglutination. Convincing evidence against this possibility is the fact that neither the absorbed streptococcal immune serum nor the absorbed sera from patients with probable streptococcal diseases were able to agglutinate sensitized streptococci. The most probable explanation is that the AAF is responsible for the agglutination of sensitized streptococci by absorbed rheumatoid arthritis sera. It is apparent that the same AAF is responsible also for the ability of nonabsorbed rheumatoid arthritis sera to agglutinate streptococci which have not been sensitized with immune serum but which are sensitized by the streptococcal agglutinins of the nonabsorbed rheumatoid arthritis serum

itself. It seems probable that we have here the same AAF which reacts with red cells sensitized with the homologous amboceptor. This opinion is borne out also by the correlation which was seen in these tests between the DAT values obtained in the Waaler-Rose test and the agglutination of sensitized streptococci. In group III, consisting of the cases of probable streptococcal diseases, there apparently is the action of the specific agglutinins produced by the streptococcal immunization, and the AAF plays at least no marked role in these cases.

The results obtained in the tests described in this chapter have suggested the following conclusions:

1. The agglutination of hemolytic streptococci by rheumatoid arthritis sera is probably not due to the presence of specific streptococcal agglutinins in these sera in greater amounts than in other sera, but rather to the presence of an agglutination activating factor which produces the agglutination of antigens sensitized with specific agglutinins.

2. The agglutination of hemolytic streptococci by rheumatoid arthritis sera cannot be regarded as evidence in support of the theory of the streptococcal etiology of rheumatoid arthritis.

Summary

Tests were carried out in an attempt to elucidate the possible role of the so-called agglutination activating factor in the agglutination of hemolytic streptococci by rheumatoid arthritis sera. The sera, all of which were able to agglutinate these streptococci, were absorbed with the same bacterium, after which they did not agglutinate streptococci. If a subagglutinating dose of a known streptococcal rabbit immune serum was added to the streptococcal antigen, the streptococci so sensitized were agglutinated by absorbed rheumatoid arthritis sera and the agglutination titer was directly proportionate to the dose of immune serum used for sensitization. On the other hand, the sensitized streptococci were not agglutinated by the absorbed sera of patients suffering from probable streptococcal diseases nor by the absorbed streptococcal immune serum of rabbit. The sera which after absorption agglutinated sensitized streptococci gave a »positive» Waaler-Rose test, whereas those which after absorption did not do so gave a »negative» test.

It is probable that the same agglutination activating factor which is responsible for the powerful capacity of rheumatoid arthritis sera to agglutinate sensitized red cells is also responsible for the agglutination of streptococci by these sera.

CHAPTER VI

DISCUSSION

It has been demonstrated by many of the earlier investigators (22, 23, 29, 30, 38, 40, 46, 49, 50, 54) that certain human sera are capable of agglutinating sheep red cells sensitized with the homologous amboceptor considerably more strongly than non-sensitized sheep cells. This phenomenon, which in this study is called the »Waaler-Rose phenomenon», the corresponding test being termed the »Waaler-Rose test», is characteristic of rheumatoid arthritis sera in particular and occurs according to the different investigators in from 23 to 80 per cent, average 50 per cent, of cases of rheumatoid arthritis. However, the phenomenon is not fully specific for rheumatoid arthritis sera, as in some cases a similar phenomenon has been found to occur with sera from patients with hepatitis, cirrhosis of liver, hepatic lesion, hyperglobulinemia, myeloma, latent syphilis, acute disseminated lupus erythematosus, osteoarthritis and chronic bronchitis (22, 23, 29, 30, 38).

The tests described in Chapter III, in which the Waaler-Rose test was performed with 1291 human sera, verified the findings of previous workers in so far that a »positive» Waaler-Rose test was obtained chiefly with rheumatoid arthritis sera and in some rare cases with other sera. In those cases which gave a »positive» Waaler-Rose test although the patient did not suffer from rheumatoid arthritis, attention was drawn to the fact that the diagnosis in a number of cases pointed to possible hepatic lesion. — According to the results obtained it seems probable that a »positive» Waaler-Rose test is sufficiently specific for rheumatoid arthritis to warrant its being accorded a certain value as a diagnostic tool. It cannot be claimed on this ground, however, that it would be of very great

practical importance in the recognition of rheumatoid arthritis. However,as the test was »positive» also in a relatively large proportion of clinically uncertain cases of rheumatoid arthritis and, on the other hand, was »negative» in most of the cases of rheumatic fever, it would seem probable that in certain cases the Waaler-Rose test may indeed assist in the establishment of diagnosis. Considering the fact that the test was not »positive» in more than 61 per cent of the clinically certain cases of rheumatoid arthritis, no great attention need be attached to a »negative» test result in this connection. According to the recently published investigations of H e l l e r *et al.* (22) it seems that the percentage of »positive» Waaler-Rose tests in rheumatoid arthritis can be considerably increased by improvement of the technique for the test. — A question important from the point of diagnostic serviceability of the Waaler-Rose test is whether the test will be »positive» already in the earliest stages of rheumatoid arthritis, when the difficulty of recognition of the disease is greatest. This question, which has not been answered in the studies made in the present work, has earlier been dealt with by H e l l e r *et al.* (22). Their findings indicated the probability that a »positive» Waaler-Rose test cannot be obtained before the stage where the X-ray discloses the characteristic joint changes. More extensive studies will be necessary on this point.

In undertaking the tests described in Chapter IV, the purpose of which was to throw more light on the nature and mode of action of the serum factor responsible for the Waaler-Rose phenomenon, particular attention was paid to the possible modes of action suggested below:

1. The action of heterophilic hemagglutinins is drawn out more strongly by a very sensitive technique.

Red cells sensitized with the homologous amboceptor undoubtedly have a greater tendency to become agglutinated than nonsensitized red cells. It is therefore comprehensible that sera containing heterophilic hemagglutinins are capable of agglutinating sensitized red cells more strongly than nonsensitized red cells. The slightly stronger agglutination of sensitized red cells by, also, most non-rheumatoid arthritis human sera is possibly to a great extent due to the action of heterophilic hemagglutinins. However, this will not serve as an adequate explanation for the very strong agglutination of sensitized red cells by rheumatoid arthritis sera as compared with

other sera. According to the observations of earlier workers (44) and those made in the present study, rheumatoid arthritis sera do not generally agglutinate nonsensitized red cells more strongly than do other human sera. It was further observed in the present study that the rheumatoid arthritis sera which have a strong agglutinating power against sensitized sheep cells have an analogous power against the patient's own sensitized red cells. This finding also speaks strongly against the concept that heterophilic hemagglutinins are responsible for the Waaler-Rose phenomenon. A further finding was that absorption with nonsensitized sheep cells removed the agglutinating power of rheumatoid arthritis sera against nonsensitized sheep cells but that no appreciable reduction occurred in the capacity of these sera to agglutinate sensitized sheep cells.

2. According to Pike *et al.* (44) the Waaler-Rose phenomenon probably is analogous, at least superficially, to the phenomenon observed by Moreschi (31), in which the sera of rabbits immunized with goat serum agglutinate rabbit cells sensitized with anti-rabbit cell goat serum, and with the phenomenon described by Coombs *et al.* (9), in which the sera of rabbits immunized with human serum agglutinate Rh positive red cells to which »incomplete» Rh antibodies have been adsorbed. As far as is known these two phenomena are due to the reaction of specific antibodies with their homologous antigens, which adhere to the surface of the red cells serving as the vehicle, and this reaction is manifested as an agglutination of the red cells.

It appears probable in the light of the test results in Chapter IV that the analogy between these two phenomena and the Waaler-Rose phenomenon indeed is superficial only. In the first place it was demonstrated that a reaction analogous to the Waaler-Rose phenomenon could also be produced by substituting homologous guinea pig amboceptor for the rabbit amboceptor when sensitizing the sheep cells. Furthermore, if in analogy to Moreschi's and Coombs's phenomena, the Waaler-Rose phenomenon were ascribed to antibodies in rheumatoid arthritis sera directed against the rabbit factor of the sensitizing serum, it could be presumed that red cells sensitized with normal rabbit serum would be agglutinated more strongly by rheumatoid arthritis sera than the corresponding nonsensitized red cells. However, this was not the case in the tests made with guinea pig, human O and sheep cells except in the case

of sheep cells sensitized with the serum of a certain rabbit. It is possible that this animal was a former amboceptor rabbit.

3. Rheumatoid arthritis sera contain a factor which activates the agglutination between the red cells and the sensitizing amboceptor in such a manner that agglutination is produced in high dilutions in which the sensitizing amboceptor alone without the presence of the activating factor is ineffective.

It appears highly probable that this is indeed the case. This explanation is in harmony with the opinions of some of the earlier investigators. Meyer (29) 1922, who, as far as is known, was the first to observe that some human sera have an unusually strong power to agglutinate red cells sensitized with the homologous amboceptor, already suggested that this phenomenon is due to a substance in these sera which intensifies specific agglutination (»Agglutinationsförndernde Substanz»). A similar opinion was held by Waaler (54) 1940 in respect to an analogous phenomenon which he was the first to observe in rheumatoid arthritis. The serum factor responsible for this phenomenon was designated by him as the »agglutination activating factor», which is the term used also in the present study.

The agglutination activating factor is of course merely a concept, which gives very little indication in a deeper sense of the occurrences which are manifested as a considerably stronger agglutination of sensitized red cells by rheumatoid arthritis sera than by other sera. In consideration, however, of the several different agglutination phenomena which earlier investigators have found to be produced by rheumatoid arthritis sera and whose interrelation has not yet been explained, it is possible that the adoption of this concept will bring us a step further in the comprehension of the peculiar agglutinating properties of the rheumatoid arthritis sera. Already *a priori* it would seem most natural that the different agglutination phenomena would be manifestations of the same peculiar property of rheumatoid arthritis sera. The concept of an agglutination activating factor seems to make it possible to find a common explanation for these different agglutination phenomena. Thus the bacterial agglutination phenomena seen in rheumatoid arthritis could be explained as follows:

The bacteria which are more strongly agglutinated by rheumatoid arthritis sera than by other sera are either inhabitants of the normal

human organism or, at least, bacteria pathogenic to man which are so common that most people presumably have undergone infection by them. As a natural consequence the sera of the majority of persons doubtlessly contain small amounts of specific agglutinins for these bacteria. In agglutination tests these weak agglutinins are not capable of producing agglutination of their homologous antigens except in the presence of the AAF. For this reason the rheumatoid arthritis sera agglutinate these bacteria and most other sera do not. The results of the tests described in Chapter V indicate that at least the capacity of rheumatoid arthritis sera to agglutinate hemolytic streptococci may probably be regarded as a result of the action of the AAF. — An opinion corresponding to this was held by W a l l i s (56, 57, 58), who suggested that the power of rheumatoid arthritis sera to agglutinate streptococci and pneumococci is a result of a peculiar capacity of these sera to intensify in a nonspecific manner the action of the specific agglutinins which already normally are present in most human sera. He was of the opinion that this property probably is related to the capacity of rheumatoid arthritis sera to agglutinate collodion particles.

In the tests described in Chapter IV it was found that the AAF exerted an action on only a part of the antigen-antibody systems studied. In the present work, as also in the experiments carried out by earlier investigators, the antigen-antibody systems in which the action of the AAF was clearly seen had in common the feature that homologous animal immune sera had been used for sensitization of the antigen.

It can be assumed from the investigations so far carried out that the AAF is contained in the β-γ-globulin fraction of the serum (46). It is known that the globulin level in rheumatoid arthritis sera is elevated (13, 38, 54). No quantitative correlation has been found between the amount of globulin and the AAF action (38, 54).

Although the nature and mode of action of the AAF have not been fully disclosed so far, our present knowledge of this factor gives some indication for speculation on the subject. The attention is drawn to the antibodies produced in connection with Rh immunization. It is known that only a part of the antibodies produced in Rh immunization are able to agglutinate Rh positive red cells in saline solution. »Incomplete» antibodies are also produced, which, it is true, adhere also in saline to the Rh positive red cells but are incapable

of agglutinating the cells except in the presence of bovine albumin. We may assume that also those immune sera with which the red cells affected by the AAF are sensitized do not contain only antibodies which produce agglutination in saline alone but also antibodies which are unable to agglutinate the antigen except in the presence of a third factor, the AAF. In this connection the AAF would thus act as a kind of »agglutination complement», which is thermostable. Such a concept would not conflict with the results obtained in the tests described in Chapter IV, in which it was found that the AAF can be adsorbed to sensitized sheep cells and released from them by elution at + 56° C.

It seems probable that the AAF is a property which is contained in the globulin fraction of normal human serum and which is present in rheumatoid arthritis serum and certain other sera in larger amounts than usually is the case. Most human sera have been found to agglutinate sensitized sheep cells in the Waaler-Rose test slightly more strongly than nonsensitized sheep cells. Although this may partly be due to the action of heterophilic hemagglutinins, the probable presence of the AAF in also these sera is indicated for instance by the fact that normal human sera absorbed with nonsensitized sheep cells agglutinated sensitized sheep cells to some degree. — It also may be mentioned that the thermostable intensifying action on bacterial agglutination and hemagglutination which the globulin fraction of certain animal sera has been found to possess (19, 39) resembles the action of the AAF in rheumatoid arthritis serum.

Thus it is possible that the occurrence of agglutination with certain sera — particularly with rheumatoid arthritis sera — is the result of a reaction between three factors, *i.e.* the antigen, the specific antibody and the AAF, and that the agglutination titer of these sera does not reflect in the first place the concentration of the specific antibody but that of the AAF. Consequently a certain degree of reservation should be employed in regarding agglutination titers of any kind as indications of the amount of specific antibodies. Secondly, the opinion expressed in 1902 by Bail (1) that agglutination phenomena, like hemolysis phenomena, are the result of the reaction of three factors — the antigen, the antibody and a factor corresponding to the complement — again becomes topical.

CHAPTER VII

CONCLUSIONS

1. The Waaler-Rose phenomenon occurs chiefly with rheumatoid arthritis sera (the Waaler-Rose test was »positive» in 61 per cent of typical cases of rheumatoid arthritis), but probably is not specific for these sera alone (the Waaler-Rose test was also »positive» in 3 per cent of cases of rheumatic fever, in 2 per cent of cases in other disease groups, and in 1 per cent of healthy persons).

2. The serum factor responsible for the Waaler-Rose phenomenon (the agglutination activating factor = AAF) does not exert an action only on sheep red cells sensitized with the homologous rabbit amboceptor but also on other red cells sensitized with their homologous immune animal amboceptors, even the patient's own sensitized cells. The AAF can be absorbed from the sera with sheep cells sensitized with the homologous rabbit amboceptor, and this absorption is the more effective the stronger the sensitization of the cells used for absorption. The AAF adsorbed to the sensitized sheep cells can be released by elution of the cells with saline solution at + 56° C.

3. It is very probable that also the capacity of rheumatoid arthritis sera to agglutinate hemolytic streptococci may be ascribed to the action of the AAF and not to the supposedly greater amount of specific streptococcal agglutinins in rheumatoid arthritis sera than in other human sera. In no case can the agglutination of streptococci by rheumatoid arthritis sera be regarded as evidence of the streptococcal etiology of rheumatoid arthritis.

BIBLIOGRAPHY

1. Bail, O.: Arch. Hyg. 1902:42:307.
2. Boots, R. H., Lipman, M. O., Coss Jr., J. A. & Ragan, C.: Proceedings of Seventh International Congress on Rheumatic Diseases. New York 1949, p. 120.
3. Bordet, J. & Gay, F. P.: Ann. Inst. Pasteur. 1906:20:467.
4. Bordet, J. & Streng, O.: Zentralbl. f. Bakt. 1909:49:260.
5. Bunim, J. J. & Mc Ewen, C.: J. Clin. Investigation 1940:19:75.
6. Cecil, R. L. & de Gara, P.: Am. J. M. Sc. 1946:211:472.
7. Cecil, R. L., Nicholls, E. E. & Stainsby, W. J.: Am. J. M. Sc. 1931:181:12.
8. Chasis, H. & Mc Ewen, C.: J. Immunol. 1936:31:439.
9. Coombs, R. R. A., Mourant, A. E. & Race, R. R.: Brit. J. Exper. Path. 1945:26:255.
10. Cox, K. E. & Hill, D. E.: Arch. Int. Med. 1934:54:27.
11. Dawidsohn, I.: J. Infect. Dis. 1933:53:219.
12. Dawidsohn, I.: J.A.M.A. 1937:108:289.
13. Davis Jr., J. S.: J. Lab. & Clin. Med. 1936:21:478.
14. Dawson, M. H. & Olmstead, M.: Proc. Soc. Exper. Biol. & Med. 1936:34:80.
15. Dawson, M. H. & Olmstead, M.: Nord. med. 1936:35:1408.
16. Dawson, M. H., Olmstead, M. & Boots, R. H.: J. Immunol. 1932:23:205.
17. Dawson, M. H., Olmstead, M. & Boots, R. H.: J. Immunol. 1932:23:187.
18. Dawson, M. H., Olmstead, M. & Jost, L.: J. Immunol. 1934: 27:355.
19. Dean, H. R.: Proc. Roy. Soc. 1911:84:416.
20. Edström, G. & Winblad, S.: Nord. med. 1947:33:506.
21. Gibson, H. J.: J. Hyg. 1930:30:337.
22. Heller, G., Jacobson, A. S. & Kolodny, M. H.: Proc. Soc. Exper. Biol. & Med. 1949:72:316.
23. Jawitz, E. & Hook, E. V.: Proc. Soc. Exper. Biol. & Med. 1949:70:650.
24. Kalbak, K.: Experimentelle og Kliniske Undersøgelser over O-streptolysin og Forekomsten af O-antistreptolysin i serum med saerlig henblik paa Forholdet ved Polyarthritis rheumatica acuta. Diss. Copenhagen 1942.
25. Kalbak, K.: Nord. med. 1944:22:1156.
26. Kalbak, K.: Nord. med. 1946:31:1997.

27. Landsteiner, K. & Miller, C. P.: J. Exper. Med. 1925:42:853
28. Mc Ewen, C., Chasis, H. & Alexander, R. C.: Proc. Soc. Exper. Biol. & Med. 1935:33:133.
29. Meyer, K.: Z. Immunit. forsch. 1922:34:229.
30. Miller, J. E., Lynch, E. R. & Landsbury, J.: J. Lab. & Clin. Med. 1949:34:1216.
31. Moreschi, C.: Zentralbl. f. Bakt. 1904:46:49.
32. Myers, W. K. & Keefer, C. S.: J. Clin. Investigation 1934:13:155.
33. Nicholls, E. E. & Stainsby, W. J.: J. Clin. Investigation. 1933:12:505.
34. Nicholls, E. E. & Stainsby, W. J.: J. Clin. Investigation 1931:10:323.
35. Oker-Blom, N.: Ann. med. exper. et biol. Fenniae 1948:26:77.
36. Oker-Blom, N.: Nord. med. 1949:41:74.
37. Olhagen, B.: Nord. med. 1947:33:509.
38. Olhagen, B.: Nord. med. 1949:42:1708.
39. Olsen, O.: Z. Immunit. forsch. 1922:33:283.
40. Ouchterlony, Ö. & Jonsson, E.: Nord. med. 1949:42:1449.
41. Packalén, T.: Nord. med. 1943:17:99.
42. Packalén, T.: Acta path. et microbiol. Scandinav. 1948:25:275.
43. Penttinen, K.: Personal report 1950.
44. Pike, R. M., Sulkin, S. E. & Goggeshall, H. C.: J. Immunol. 1949:63:447.
45. Porsman, V. A.: Nord. med. 1948:40:2147.
46. Rose, H. M., Ragan, C., Pearce, E. & Lipman, M. O.: Proc. Soc. Exper. Biol. & Med. 1948:68:1.
47. Short, C. L., Dienes, L. & Bauer, W.: J.A.M.A. 1937:108:2087.
48. Streng, O.: Z. Immunit. forsch. 1909:2:415.
49. Sulkin, S. E., Pike, R. M. & Goggeshall, H. C.: Proc. Soc. Exper. Biol. & Med. 1949:70:475.
50. Swartz, N. & Schlossmann, K.: Nord. med. 1949:42:1390.
51. Thulin, K. E.: Nord. med. 1947:33:508.
52. Thulin, K. E.: Serological Aspects of Hemolytic Streptococci with Special Reference to the Occurrence of O, K, and L Antigens, and some Clinical Applications. Diss. Lund. 1948.
53. Thulin, K. E. & Vahlne, G.: Acta path. et microbiol. Scandinav. 1946:23:484.
54. Waaler, E.: Acta path. et microbiol. Scandinav. 1940:17:172.
55. Wager, O.: Ann. med exper. et biol. Fenniae 1950:28:154.
56. Wallis, A. D.: Am. J. M. Sc. 1946:212:716.
57. Wallis, A. D.: Am. J. M. Sc. 1946:212:718.
58. Wallis, A. D.: Am. J. M. Sc. 1947:213:94.
59. Winblad, S. & Edström, G.: Acta path. et microbiol. Scandinav. 1948:25:715.